Clinical Studies

Cancer Research

Plastin3 Is a Novel Marker for Circulating Tumor Cells Undergoing the Epithelial–Mesenchymal Transition and Is Associated with Colorectal Cancer Prognosis

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Abstract

Circulating tumor cells (CTC) in blood have attracted attention both as potential seeds for metastasis and as biomarkers. However, most CTC detection systems might miss epithelial–mesenchymal transition (EMT)-induced metastatic cells because detection is based on epithelial markers. First, to discover novel markers capable of detecting CTCs in which EMT has not been repressed, microarray analysis of 132 colorectal cancers (CRC) from Japanese patients was conducted, and 2,969 genes were detected that were overexpressed relative to normal colon mucosa. From the detected genes, we selected those that were overexpressed CRC with distant metastasis. Then, we analyzed the CRC metastasis-specific genes ($n = 22$) to determine whether they were expressed in normal circulation. As a result, *PLS3* was discovered as a CTC marker that was expressed in metastatic CRC cells but not in normal circulation. Using fluorescent immunocytochemistry, we validated that *PLS3* was expressed in EMT-induced CTC in peripheral blood from patients with CRC with distant metastasis. *PLS3*-expressing cells were detected in the peripheral blood of approximately one-third of an independent set of 711 Japanese patients with CRC. Multivariate analysis showed that *PLS3*-positive CTC was independently associated with prognosis in the training set ($n = 381$) and the validation set [$n = 330$; HR = 2.17; 95% confidence interval (CI) = 1.38–3.40 and HR = 3.92; 95% CI = 2.27–6.85]. The association between *PLS3*-positive CTC and prognosis was particularly strong in patients with Dukes B (HR = 4.07; 95% CI = 1.50–11.57) and Dukes C (HR = 2.57; 95% CI = 1.42–4.63). *PLS3* is a novel marker for metastatic CRC cells, and it possesses significant prognostic value. *Cancer Res; 73(7); 2059–69.* ©*2012 AACR.*

Introduction

Small populations of cancer cells in the circulatory system that have detached from the primary tumor are designated as circulating tumor cells (CTC; refs. 1, 2). Many research groups have developed sophisticated physical devices to capture CTCs (3). Antibodies against epithelial cell adhesion molecule (EpCAM) and cytokeratins are commonly used to capture and detect CTCs, such as in the U.S. Food and Drug Administration–approved CellSearch system (1, 4–8) and the new CTC-chip system (9–11). However, these approaches are potentially hampered by the fact that migratory cancer cells may undergo a process called the epithelial–mesenchymal transition (EMT) that is characterized by downregulation of epithelial markers, including cytokeratins and EpCAM (12–16).

Colorectal cancer (CRC) is one of the most frequent malignancies in industrialized countries, and metastasis to distant

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organs (e.g., liver and lung) is the leading cause of death (17, 18). Many groups have reported that CTC detection using real-time PCR (RT-PCR) or immunologic techniques was associated with CRC progression and poor prognosis (3, 20–22). Rahbari and colleagues conducted a meta-analysis of 36 studies that included 3,094 patients with CRC to determine the clinical significance of CTC/disseminated tumor cells in the circulation. That analysis showed that the detection of CTCs was associated with decreased overall survival in stage I to III patients with CRC (HR = 1.64; 95% CI = 0.79–3.42) and in stage I to IV patients with CRC (HR = 2.76; 95% CI = 1.73–4.41; ref. 19). Moreover, that meta-analysis suggested that CTC detection was a better prognosticator in advanced CRC than in early CRC. Therefore, it is important to discover new CTC markers especially for patients with early CRC.

Recent reports have indicated the presence of post-EMT CTCs in patients with various types of epithelial tumors (20, 21). Therefore, it is important to discover new CTC markers that are not suppressed by the induction of EMT in CTC. The purpose of this study was to find a new marker for CTCs that is not repressed during EMT and to evaluate the prognostic significance of this marker in CRC. On the basis of comparative microarray analyses, we identified *Plastin3* (*PLS3*), which codes for an actin-bundling protein known to inhibit cofilin-mediated depolymerization of actin fibers (22, 23). Then, we showed the clinical use of *PLS3* as a suitable new marker for CTCs in patients with CRC, especially in early-stage patients (Dukes B) without lymph node metastases and no evidence of overt metastases by current imaging procedures.

Materials and Methods

Overview of the strategy

First, to discover novel markers capable of detecting CTCs with metastatic ability, we conducted microarray analysis of CRC to identify overexpressed genes. Among the detected genes, we selected those that were highly expressed in CRC with distant metastasis compared with those without distant metastasis. Then, we analyzed the CRC metastasis-specific genes to determine whether they were expressed in normal circulation (24). As a result, *PLS3* was discovered as a CTC marker that was expressed in metastatic CRC cells but not in normal circulation. We then determined whether *PLS3* was expressed at higher levels in metastatic CRC samples than normal colon mucosa in an independent group of primary CRC tissues. Finally, we assessed the clinical significance of *PLS3* expression in preoperative peripheral blood in independent training and validation sets. All clinical CRC samples in this study were collected in Japan and were used in accordance with Institutional guidelines and the Helsinki Declaration after obtaining written informed consent from all the participants.

Microarray data analysis

Tissues from a series of 132 patients with CRC were collected by laser microdissection with the Leica Laser Microdissection System (Leica Microsystems), as previously described (25). All patients underwent resection of the primary tumor at Kyushu University Hospital (Beppu, Japan) or their affiliated hospitals from 2000 to 2008. The average age of the patients was 64.6 years, and approximately 56% were male and 46% were female. As for clinicopathologic factors, tumor stages T1, T2, T3, and T4 were distributed as follows: 4.6%, 25.2%, 52.3%, and 17.9%, respectively. In this patient population, 46% were positive for lymph node metastasis and 12% were positive for distant metastasis. For gene expression analyses, we used the commercially available Human Whole Genome Oligo DNA Microarray Kit (Agilent Technologies). Labeled cRNAs were fragmented and hybridized to an oligonucleotide microarray (Whole Human Genome 4 × 44K Agilent G4112F). Fluorescence intensities were determined with an Agilent DNA Microarray Scanner and were analyzed using G2567AA Feature Extraction Software, version A.7.5.1 (Agilent Technologies) that used the locally weighted linear regression curve fit (LOWESS) normalization method (26). This microarray study followed the Minimum Information About a Microarray Experiment guidelines issued by the Microarray Gene Expression Data group (27). Further analyses were conducted using the GeneSpring, version 7.3 (Silicon Genetics). Our microarray data were submitted to a public repository, the Gene Expression Omnibus (GEO). The accession number is GSE21815.

Gene set enrichment analysis

The associations between *PLS3* expression and previously curated gene expression signatures were analyzed by applying gene set enrichment analysis (GSEA; ref.28) to the expression profiles of 950 human multiple cancer cell lines stored in ArrayExpress (EMTAB-37). RNA expression data of these cell lines were measured by Affymetrix GeneChip Human Genome U133 Plus 2.0 and normalized with the dChip method (29). To collapse each probe set on the array to a single gene, the probe with the highest variance among multiple probes that corresponded to the same gene was selected, and the probes that mapped to transcripts from unknown genes were removed, which produced a 20,647 (genes) x 950 (cancer cell lines) gene expression matrix. To identify gene expression signatures that were highly correlated with *PLS3* expression, GSEA was conducted for 2,026 curated gene sets in a molecular signature database (28–30) and an EMT-related gene set that was overexpressed in the "mesenchymal" subclass as compared with "proneural" and "proliferative" subclasses of high-grade glioma (31, 32). The metric for ranking genes in GSEA was the Pearson correlation coefficient with *PLS3* expression and the number of permutations for calculating *P* values was 1,000.

CRC clinical samples for survival analyses

All peripheral blood samples were collected from patients with CRC at Teikyo University Hospital (Tokyo, Japan), Kyushu University Hospital, or their affiliated hospitals, and they differed from the patients used for microarray and RT-PCR studies of primary CRC. Between 2000 and 2004, peripheral blood samples were obtained before surgery as a training set ($n = 381$). The average age was 66.76 ± 11.02, and approximately 57% were male and 43% were female. Patients with Dukes A, B, C, and D were distributed as follows: approximately 13%, 40%, 34%, and 13%, respectively, in the training set. Between 2005 and 2008, we collected peripheral blood samples as a validation set ($n = 330$). The average age was 67.51 ± 11.08,

and approximately 61% were male and 39% were female. The distribution of patients with Dukes A, B, C, and D was approximately 11%, 48%, 31%, and 10%, respectively, in the validation set (Supplementary Table S1). These samples were used in accordance with Institutional guidelines and the Helsinki Declaration after obtaining written informed consent from all participants. Total RNA was extracted from peripheral blood using a PAXgene Blood RNA Kit (Qiagen), as previously described (33). Control peripheral blood samples were collected from 25 healthy volunteers; their average age was 52 years, and 60% were male and 40% were female. They had no evidence of any disease by physical examination, blood tests, X-ray, and colonoscopy.

The number of CRC cases with data on recurrence ($n = 628$) is less than the total number of cases ($n = 711$) because patients with Dukes D CRC ($n = 83$) already had metastases at preoperative diagnosis. To analyze the development of subsequent relapse after curative resection, these patients were excluded from this analysis (Table 1).

Postoperative follow-up was conducted along the guidelines published by the Japanese Society for Cancer of the Colon and Rectum. Patients with Dukes stage A to C were evaluated for tumor recurrence as follows. Physical examination and tumor marker (CEA and CA19-9) testing was conducted every 3 months for 3 years, and then every 6 months for 5 years. To evaluate recurrence, a computed tomography (CT) or MRI scan was repeated every 6 to 12 weeks for 3 years, and then 6 months for up to 5 years after surgery. Colon evaluation, including colonoscopy or colon radiography, was conducted every 2 years or annually for 3 years. Patients with Dukes stage D and mucosal cancer without lymph node metastasis did not follow these surveillance protocols completely. However, confirmation of recurrence in all patients was required to evaluate imaging or pathologic diagnosis.

Table 1. Relationship between peripheral blood *PLS3* and clinicopathologic factors in 711 patients with CRC in Teikyo University and Kyushu University in 2000 to 2008

Characteristics		PLS3 positive $n = 179$ (%)	PLS3 negative $n = 532$ (%)	*P*
Sex	Female	84 (46.9%)	208 (39.1%)	0.07
	Male	95 (53.1%)	324 (60.9%)	
Tumor size	<5 cm	111 (62.0%)	299 (56.2%)	0.17
	≥5 cm	68 (38.0%)	233 (43.8%)	
Depth of invasion	<pT3	14 (7.8%)	96 (18.0%)	0.001
	≥pT3	165 (92.2%)	436 (82.0%)	
Lymphatic invasion	−	104 (58.1%)	349 (65.6%)	0.07
	+	75 (41.9%)	183 (34.4%)	
Venous invasion	−	62 (34.6%)	218 (41.0%)	0.13
	+	117 (65.4%)	314 (59.0%)	
Lymph node metastasis	−	84 (46.9%)	335 (63.0%)	0.0001
	+	95 (53.1%)	197 (37.0%)	
Tumor grade	Well-differentiated	137 (76.5%)	382 (71.8%)	0.22
	Non well-differentiated	42 (23.5%)	150 (28.2%)	
Liver metastasis	−	153 (85.5%)	497 (93.4%)	0.001
	+	26 (14.5%)	35 (6.6%)	
Peritoneal dissemination	−	169 (94.4%)	522 (98.1%)	0.009
	+	10 (5.6%)	10 (1.9%)	
Serum CEA	≥5 ng/mL	92 (51.7%)	287 (53.9%)	0.62
	>5 ng/mL	86 (48.3%)	245 (46.1%)	
Adjuvant Chemotherapy ($n = 628$)	−	72 (50.7%)	322 (66.3%)	0.0007
	+	70 (49.3%)	164 (33.7%)	
Recurrence ($n = 628$)	−	74 (50.7%)	411 (85.3%)	<0.0001
	+	72 (49.3%)	71 (14.7%)	
Dukes stage	A	10 (5.6%)	75 (14.1%)	0.0001
	B	68 (38.0%)	241 (45.3%)	
	C	68 (38.0%)	166 (31.2%)	
	D	33 (18.4%)	50 (9.4%)	
Cause of death ($n = 174$)	Cancer death	79 (89.8%)	82 (95.3%)	0.16
	Noncancer death	9 (10.2%)	4 (4.7%)	

Abbreviation: CEA, carcinoembryonic antigen.

A second independent set of 110 nonlaser microdissected primary CRC samples were obtained from Kyushu University Hospital or their affiliated hospitals. Total RNA extraction and cDNA synthesis were conducted as previously described (34).

Quantitative RT-PCR

Gene-specific oligonucleotide primers were designed for PCR. The following primers were used: the *PLS3* sense primer, 5′-CCTTCCGTAACTGGATGAACTC-3′ and antisense primer, 5′-GGATGCTTCCCTAATTCAACAG-3′ and the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) sense primer, 5′-TTGGTATCGTGGAAGGACTCA-3′, and antisense primer, 5′-TGTCATCATATTTGGCAGGTT-3′. PCR amplification was conducted in a LightCycler (Roche) using the LightCycler-FastStart DNA Master SYBR Green I Kit, as previously described (34).

Cell sorting

We obtained 10 mL of heparinized peripheral blood from 3 recurrent patients with colon cancer with liver metastasis. Blood mononuclear cells were obtained by Ficoll density centrifugation at 1,800 rpm for 25 minutes. Erythrocytes were lysed with ammonium chloride buffer (BD Pharm Lyse, BD Biosciences). Cell sorting was conducted by a magnetic cell sorting system (autoMACS, Miltenyi Biotec) using magnetically labeled anti-CD45 microbeads and anti-CD326 (EpCAM; Miltenyi Biotec). Sorted cells were maintained in RPMI-1640 containing 10% FBS. The cells were cultured for 24 hours in a humidified 5% CO_2 incubator at 37°C before fluorescent immunocytochemistry.

Fluorescent immunocytochemical analysis of CTCs

Sorted cells were seeded on glass coverslips and incubated for 24 hours at 37°C. After washing with PBS to exclude nonattached circulating cells, such as lymphocytes, the cells were fixed with 90% methanol (−20°C) for 5 minutes, followed by incubation with mouse anti-cytokeratin (1:100; clone MNF-116, DAKO), goat anti-PLS3 antibodies (1:100; sc-16655, Santa Cruz Biotechnology), and rabbit anti-vimentin antibodies (1:600; ab45939, Abcam) for 1 hour at room temperature. To detect antibodies against cytokeratin, PLS3, and vimentin, fluorophore-labeled antibodies with anti-mouse, anti-goat, and anti-rabbit specificities (A-11060, A-21050, and A-10040; Molecular Probes) were used for 1 hour at room temperature at a dilution of 1:2,000. All sections were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Cellular fluorescence was then visualized with a confocal LSM510 microscope (Carl Zeiss Microimaging) at × 63 magnification.

Statistical analysis

The sample sizes of the training and validation sets were calculated using SAS statistics software version 9 (SAS Inst. Inc.) based on the results of a small scale test ($n = 50$) under which the one-sided P value of 0.025 would have 80% power to detect a difference between the disease-free survival and overall survival curves of patients with and without CTC. The sample size refers to both the training and validation sets. The cut-off values of *PLS3* were determined by receiver operating characteristic (ROC) curves, which were constructed by plotting all possible sensitivity/1−specificity pairs in the training set. Sensitivities of *PLS3* were calculated as the ratio of the number of patients with PCR evidence of *PLS3* in peripheral blood divided by the number of patients who had metastasis. Specificities were calculated as the ratio of the number of patients without PCR evidence of *PLS3* in peripheral blood divided by the number of patients who did not have metastasis. Metastasis was defined by imaging analysis (CT or MRI). ROC analysis and the optimal cut-off value were calculated as the *PLS3* level that maximized the sensitivity/(1−specificity) as previously published (30). The relationships between overall survival rate, disease-free survival rate, and *PLS3* were analyzed by Kaplan–Meier survival curves and the log-rank tests. Cox proportional hazards regression was used to determine multivariate HRs for the overall survival rate and the disease-free survival rate. The comparison of clinicopathologic factors was analyzed using the Student t test, χ^2 tests, and ANOVA. All P values were 2-sided, and $P < 0.05$ was considered statistically significant. Data were analyzed using JMP software v. 7 (SAS Inst. Inc.).

Results

Identification of PLS3 as a new CTC marker by microarray analysis

Microarray analysis showed that *PLS3* was a novel CTC marker: it was expressed at high levels in metastatic CRC cells but was not expressed in normal blood cells (Fig. 1A). Little is currently known about *PLS3*. Thus, it was necessary to focus on the role of *PLS3* in oncogenesis and/or the progression of CRC. We conducted GSEA *in silico* to validate our hypothesis that the *PLS3* gene was significantly associated with the EMT gene set and/or EMT-related molecules. In addition, we sought to identify gene sets that were significantly related to the *PLS3* gene expression profile. Functional gene sets correlated with *PLS3* expression were analyzed by GSEA. GSEA showed that genes that were highly correlated (Pearson correlation) with *PLS3* expression were significantly enriched in the *TGF-β*–specific gene expression signature [$P < 0.001$ and false discovery rate (FDR) = 0.001], the metastasis gene expression signature ($P < 0.001$ and FDR = 0.001), the stemness gene expression signature ($P < 0.001$ and FDR = 0.001), and the mesenchymal gene expression signature ($P < 0.001$ and FDR $\leq$ 0.001; Fig. 1B and C and Supplementary Table S2).

We further examined the distribution of *PLS3* gene expression using a human cDNA tissue panel (Takara Bio Inc.), peripheral blood from healthy volunteers and 2 GEO databases (GDS596 and GDS3113; refs. 24, 35). *PLS3* was not expressed in peripheral blood or bone marrow in any of these sources (Supplementary Fig. S3).

To validate *PLS3* as a potential CTC marker, we examined mRNA-expression levels of *PLS3* in a second independent set of nonlaser microdissected primary CRC samples. RT-PCR was used to define *PLS3* expression in CRC tissues and corresponding normal tissues ($n = 110$). *PLS3* expression levels in tumors were significantly higher than in normal tissues ($P < 0.001$; Fig. 2A). Immunohistochemical staining confirmed that PLS3 protein was expressed at higher levels in clinical CRC tissues than

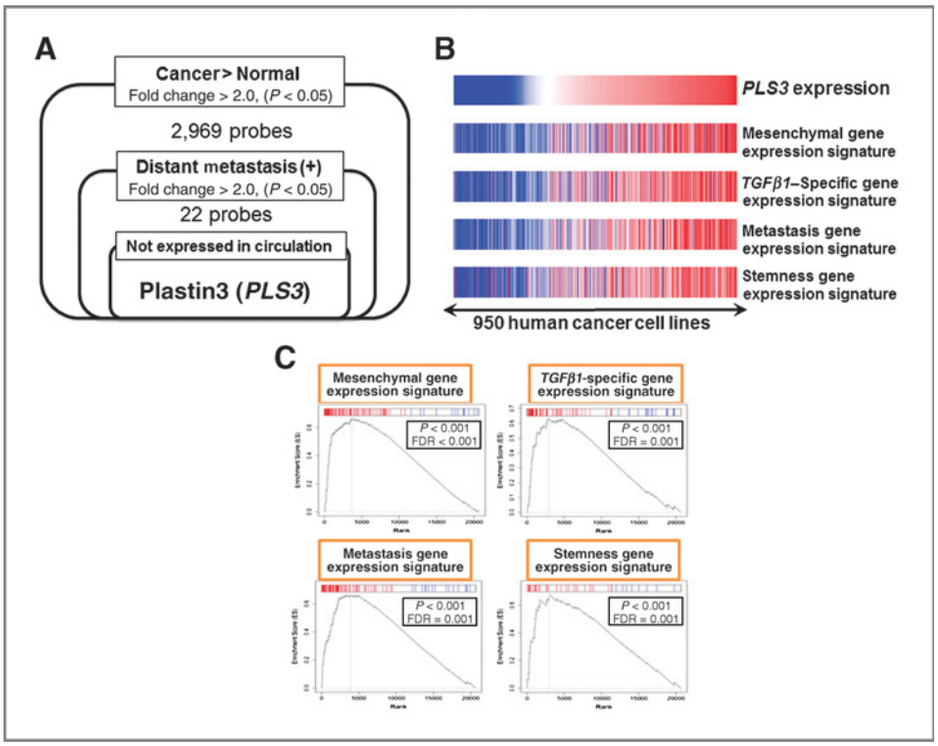

Figure 1. Strategy for the identification of a candidate marker PLS3. A, diagram of the strategy leading to the discovery of a candidate CTC marker gene, *PLS3*, which is highly expressed in cancer tissue with distant metastasis, but not in blood- and bone marrow–derived cells. This gene was detected by both our microarray analysis and the GEO database analysis. B, heatmap of the gene expression averages of 29 different cancers enriched in mesenchymal, *TGFβ-1*–specific, metastasis, and stemness gene expression signatures. The mesenchymal gene expression signature was depicted as the set of genes that are overexpressed in mesenchymal-type cells. The enriched gene expression averages were rank-sorted according to the order of *PLS3* expressions. The red and blue colors indicate relatively low and high expression, respectively. C, enrichment plots of mesenchymal, *TGFβ-1*–specific, metastasis, and stemness gene expression signatures sorted according to the correlations with *PLS3* expression. The bar-code plot indicates the position of the genes in each gene set; red and blue colors represent positive and negative Pearson correlation with *PLS3* expression, respectively.

in normal tissues (Supplementary Fig. S1). Within the CRC cohort, *PLS3* expression in tumors from patients with Dukes D with overt metastases in distant organs exhibited higher levels of *PLS3* expression than a group of patients with Dukes A to C without such metastases ($P < 0.05$; Fig. 2B). Moreover, the *PLS3* high-expression group was more likely to have unfavorable clinicopathologic features and poorer prognoses than the low-expression group (Supplementary Fig. S2A to S2C).

PLS3 expression in EMT-induced cancer cells

EMT was induced in CaR-1 cells by TGFβ-1 stimulation (Supplementary Fig. S4A). *PLS3* expression was not downregulated in EMT-induced CaR-1 cells. Interestingly, levels of *E-cadherin*, an epithelial marker, were downregulated in EMT-induced CaR-1 cells; however, *PLS3* and *vimentin* levels were upregulated in a parallel manner in these cells (Supplementary Fig. S4B). Moreover, we examined *PLS3* expression in the colon cancer cell line LoVo expressing CD133, CD44, and EPHB2, a putative marker for colon cancer stem cells. The results showed that the expression level of *PLS3* in LoVo-expressing stem cell markers was not repressed (Supplementary Fig. S5).

To confirm that *PLS3* can be used to detect EMT-induced CTCs in patients with cancer, we examined the relationship between EpCAM, cytokeratin, vimentin, and PLS3 protein expression in CTCs by fluorescence immunocytochemistry. We showed that PLS3 is expressed at the protein level in CTCs by using the autoMACS (Miltenyi Biotec) to sort cells expressing EpCAM (+)/(−) and CD45 (+)/(−). CTCs expressing cytokeratins were detected not only in EpCAM (+)/CD45 (−) cells but also in EpCAM (−)/CD45 (−) cells. PLS3 expression in all cytokeratin (+) CTCs was detected in both EpCAM (+) and EpCAM (−) cells (Fig. 3). Moreover, PLS3 was also expressed on CTCs that displayed reduced cytokeratin staining, accompanied by strong staining for vimentin, the mesenchymal intermediate filament. To exclude the possibility that these cells were contaminated by residual CD45 (+) blood cells present after CD45 depletion (36), we stained peripheral blood mononuclear cells (PBMC) from healthy volunteers using fluorescence immunocytochemistry. PLS3 was expressed on all CaR-1 rectal cancer cells used as positive controls, but PLS3 staining was consistently absent on all PBMC (Supplementary Fig. S6).

Association of PLS3-positive CTCs with clinicopathologic risk factors

Clinicopathologic analysis of *PLS3* expression in peripheral blood samples from 711 patients with CRC revealed that

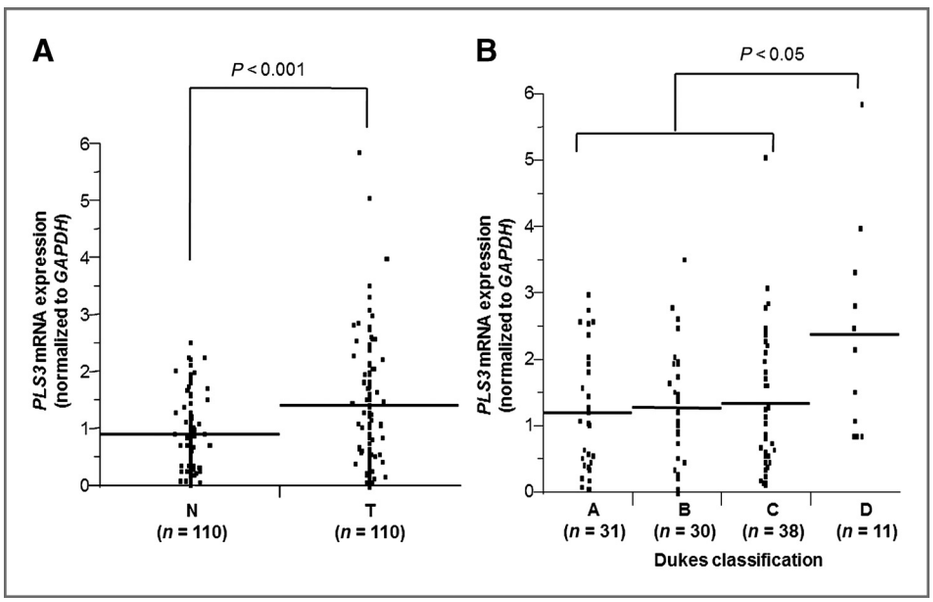

Figure 2. Validation of microarray data with a second independent set of 110 nonlaser-microdissected primary CRC samples. A, *PLS3* mRNA expression in cancerous (T) and noncancerous (N) tissues from patients with CRC determined by RT-PCR. T group ($n = 110$), *PLS3* mRNA (T)/*GAPDH* mRNA (T); N group ($n = 110$), *PLS3* mRNA (N)/*GAPDH* mRNA (N; $P < 0.001$). B, concordant expression of *PLS3* with Dukes classification. *PLS3* mRNA expression in primary CRC samples from Dukes stage A ($n = 31$), Dukes stage B ($n = 30$), Dukes stage C ($n = 38$), and Dukes stage D ($n = 11$) groups was determined by RT-PCR. All data were normalized to *GAPDH*. Horizontal lines indicate the mean expression levels of *PLS3*. The difference between groups was analyzed with ANOVA ($P = 0.0126$) and multiple comparison tests ($P < 0.05$).

PLS3-positive expression in peripheral blood ($n = 179$; 33.6%) was associated with a greater depth of invasion ($P = 0.001$), lymph node metastasis ($P = 0.0001$), liver metastasis ($P = 0.001$), peritoneal dissemination ($P = 0.009$), recurrence rate ($P < 0.0001$), and Dukes staging progression ($P = 0.0001$; Table 1).

Prognostic relevance of PLS3-positive CTCs

Peripheral blood samples sets were used to clarify the prognostic values of the new CTC marker *PLS3* (training sample set, $n = 381$; validation sample set, $n = 330$: total samples $n = 711$; Supplementary Table S1). Patients with

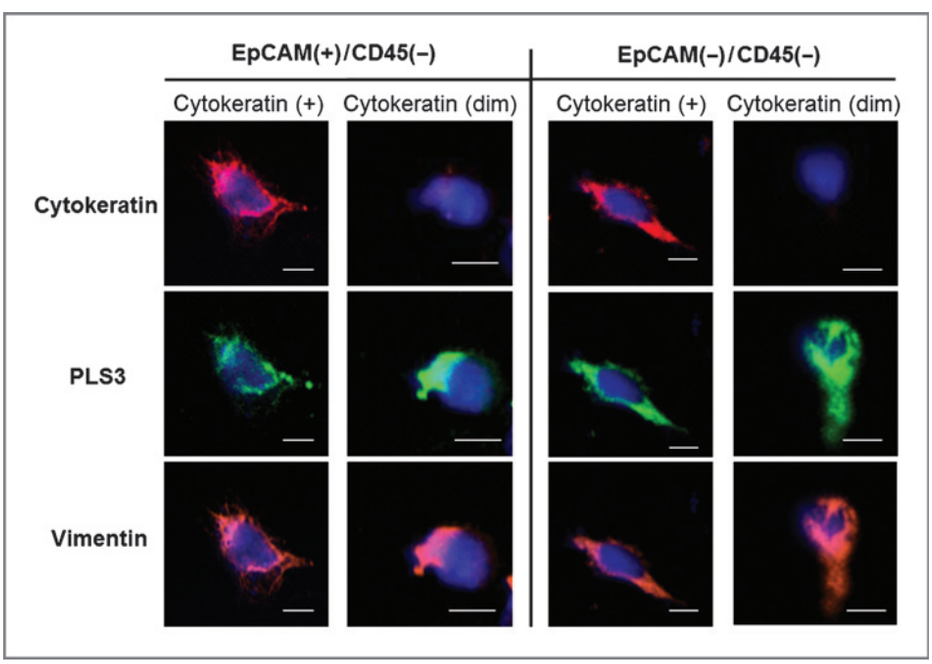

Figure 3. Immunocytochemical staining of PLS3 in circulating CRC cells. Images of EpCAM(+)/CD45 (–) or EpCAM(–)/CD45 (–) circulating tumor cells immunostained with antibodies against cytokeratin (red, top), PLS3 (green, middle), and vimentin (orange, bottom). All sections were counterstained with DAPI (blue; original magnification, ×63; scale bar, 10 μm).

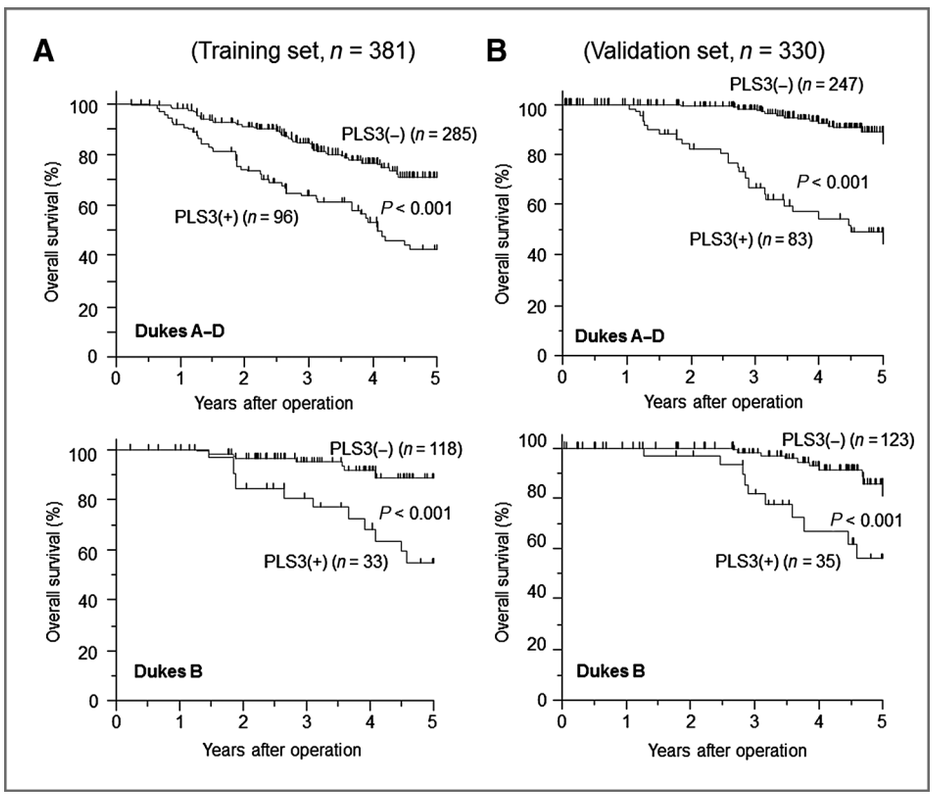

Figure 4. Survival curves of patients with CRC based on the level of PLS3 mRNA expression in peripheral blood from training set ($n = 381$, 2000–2004) and validation set ($n = 330$, 2005–2008). A, training set: overall survival curves of patients with CRC (Dukes A–D, $n = 381$) based on the level of PLS3 expression in peripheral blood ($P < 0.001$). Overall survival curve of patients with Dukes B CRC ($n = 151$) based on the level of PLS3 expression in peripheral blood ($P < 0.001$). B, validation set: overall survival curves of patients with CRC (Dukes A–D, $n = 330$) based on the level of PLS3 expression in peripheral blood ($P < 0.001$). Overall survival curve of patients with Dukes B CRC ($n = 158$) based on the level of PLS3 expression in peripheral blood ($P < 0.001$). The cut-off values of PLS3 were determined by ROC curves, which were constructed by plotting all possible sensitivity/1−specificity pairs in the training sets.

PLS3-positive CTCs in peripheral blood from each of the Dukes stages had a significantly shorter overall survival than patients without these cells (Dukes A–D, $P < 0.001$; Dukes B, $P < 0.001$; Fig. 4A, training set). Examining only patients with CRC without synchronous distant metastasis (Dukes A–C, $n = 332$), patients with *PLS3*-positive CTCs in peripheral blood from each Dukes stage had significantly shorter disease-free survival than *PLS3*-negative patients (data not shown). Multivariate analysis showed that *PLS3*-positive CTCs in peripheral blood are independently associated with poor prognosis (HR = 2.17; 95% CI = 1.38–3.40) and recurrence (HR = 2.32; 95% CI = 1.42–3.74; Table 2).

Because of the high clinical relevance of finding new prognostic factors in patients with Dukes B and C, we analyzed this subgroup separately. Interestingly, other existing clinicopathologic factors were not significantly and independently associated with poor prognosis and recurrence in these subgroups, whereas the detection of *PLS3*-positive CTCs in peripheral blood remained prognostically significant with regard to both overall survival and disease-free survival (Table 3; Dukes B, HR = 4.07; 95% CI = 1.50–11.57 and HR = 2.73; 95% CI = 1.16–6.24; Dukes C, HR = 2.57; 95% CI = 1.42–4.63 and HR = 2.19; 95% CI = 1.10–4.34). Clinical significance of *PLS3* expression in the training sample set ($n = 381$) was validated in an independent validation sample set ($n = 330$; Fig. 4B and Table 2 and 3). To validate the ability of *PLS3* expression to detect CTCs after recurrence, we evaluated *PLS3* expression in available peripheral blood of recurrent patients ($n = 10$) and nonrecurrent patients ($n = 25$). *PLS3* expression was detected in all recurrent patients at statistically significantly higher levels than before recurrence and in comparison with patients with CRC who had not experienced recurrence (Supplementary Fig. S7).

To show that *PLS3* is a better marker in CRC than cytokeratins, the current markers for CTC (3), we also conducted RT-PCR analysis of peripheral blood samples from patients with CRC using cytokeratin 19 and 20 transcripts frequently used for CTC detection in CRC and other solid tumors (1). In contrast to our findings with *PLS3*, cytokeratin 19 and 20 transcripts were not associated with recurrence in patients with Dukes B (Supplementary Fig. S8).

We further examined the expression of *PLS3* by RT-PCR in several cancer cell lines derived from other tumor entities, including esophageal cancer, gastric cancer, liver tumor, pancreatic cancer, breast cancer, lung cancer, prostate cancer, melanoma, and hematopoietic malignancies. The expression of *PLS3* was detected in all solid cancer cell lines, but not in cell lines derived from hematopoietic malignancies (Supplementary Fig. S9).

Discussion

Detection of CTCs has gained considerable attention in recent years. However, the current markers used for the enrichment and detection of these cells may miss the most aggressive subpopulations of EMT-induced CTCs (37). This

Table 2. Multivariate analysis of the association with overall and disease-free survival of CRC patients in Teikyo University and Kyushu University in 2000 to 2008

	Training set ($n = 381$)		Validation set ($n = 330$)	
	Multivariate analysis for overall survival	Multivariate analysis for disease-free survival	Multivariate analysis for overall survival	Multivariate analysis for disease-free survival
Characteristics	HR (95% CI)	HR (95% CI)	HR (95% CI)	HR (95% CI)
PLS3 in PB	2.17 (1.38–3.40)	2.32 (1.42–3.74)	3.92 (2.27–6.85)	4.63 (2.69–7.94)
Dukes stage	4.33 (2.98–6.18)	1.51 (1.26–2.87)	3.74 (2.44–5.72)	1.91 (1.10–3.46)
Depth of invasion	1.09 (0.45–3.24)	2.08 (0.81–6.22)	1.06 (0.37–4.48)	3.31 (0.71–5.90)
Lymph node metastasis	0.47 (0.28–0.82)	1.61 (0.77–1.83)	0.83 (0.46–1.50)	0.89 (0.59–2.07)
Lymphatic invasion	1.26 (0.79–2.02)	1.15 (0.70–1.87)	1.23 (0.69–2.21)	1.38 (0.78–2.48)
Venous invasion	1.80 (1.05–3.22)	1.37 (0.82–2.33)	2.56 (1.09–7.08)	1.35 (0.73–2.58)
CEA	1.64 (1.04–2.60)	1.74 (1.10–2.78)	1.42 (0.82–2.50)	1.21 (0.72–2.02)
Age	2.05 (0.63–7.04)	1.02 (0.35–3.08)	1.02 (0.59–1.05)	1.00 (0.97–1.03)
Gender	1.16 (0.75–1.82)	1.25 (0.79–2.01)	1.49 (0.85–2.67)	0.29 (0.14–2.11)

NOTE: Dukes stages, A, B, C or D; depth of invasion, muscularis propria invasion, positive or negative; lymphatic invasion, positive or negative; lymph node metastasis, positive or negative; venous invasion, positive or negative; histologic type, well differentiated or moderately and poorly differentiated and mucinous types; CEA, CEA expression, positive or negative in peripheral blood.
Abbreviations: PB, peripheral blood; CEA, carcinoembryonic antigen.

study showed that *PLS3* is a novel CTC marker that continues to be expressed during EMT. *PLS3* can also be used to identify tumor cells with downregulated expression of epithelial antigens currently used for capturing CTCs (i.e., EpCAM and cytokeratins). Moreover, the detection of *PLS3*-positive CTCs was an independent prognostic factor in patients with CRC.Thus, the future use of *PLS3* as a marker for CTCs may overcome some of the limitations of current CTC assays and contribute to improved staging of patients with CRC.

Little is known about the biology of *PLS3* and its relevance to solid cancer development and progression. The *PLS3* gene, located on chromosome Xq23, functions to polymerize actin fibers through inhibition of *cofilin*-mediated actin depolymerization (38). It is known that cisplatin- or UV-resistant cancer cell lines overexpress *PLS3* (39, 40), and CRC lines overexpressing *PLS3* acquire invasiveness through downregulation of *E-cadherin* (41). Moreover, the GSEA (public database) results provided supportive evidence that *PLS3* might be involved in EMT via TGFβ-1 stimulation and that *PLS3* was related to CRC metastasis and cancer stemness as well. Because the GSEA findings were independent of our own microarray data, it strongly suggested that *PLS3* might play an important role in EMT in CRC cases. However, there is nothing known about the clinical significance of *PLS3* in epithelial malignancies, including CRC. This study provides the first evidence for the clinical significance of *PLS3* in primary CRC and shows that it is a novel marker for CTC.

In the present study, we conducted an extensive validation of the clinical use of *PLS3* as a novel marker for CTCs. Although many groups have developed new assays for CTC detection (37, 42), an in-depth clinical validation showing the prognostic significance of the cells captured by these assays is still lacking (3). Linking the CTC assay results to the development of metastasis and survival in a large cohort of patients with cancer is crucial to show that the assay provides clinically relevant information. Our present analysis of a large set of patients with CRC revealed that the presence of *PLS3*-positive CTCs was an independent prognostic factor with regard to relapse and survival. This finding is consistent with the view that the presence of CTCs in peripheral blood is an indicator of metastatic spread. Further subset analyses showed that CTC detection by *PLS3* was the only significant prognostic factor in patients with Dukes B. This finding is of utmost importance, as it shows that early tumor cell dissemination can be detected by the established *PLS3* assay and is relevant for the subsequent course of the disease in patients with CRC. Moreover, *PLS3* was expressed in several cancer cell lines, but not in hematopoietic malignancies or peripheral blood from healthy volunteers. Our data indicate that *PLS3* may be a useful CTC marker in a broad range of solid tumors.

It is currently unclear whether patients with Dukes B profit from adjuvant chemotherapy. We hypothesize that patients with disseminated disease may receive the greatest benefits from systemic therapies aimed at preventing metastatic relapse. Moreover, if patients with low recurrent rates can be detected by *PLS3* expression in peripheral blood, adjuvant chemotherapy for some patients with Dukes C may be unnecessary. These hypotheses can now be tested in future clinical trials using our *PLS3* assay for stratification (and even monitoring) of therapy, evaluating whether patients with CRC with *PLS3*-positive CTCs might specifically profit from additional adjuvant therapies.

In conclusion, the high expression of *PLS3* in peripheral blood was independently associated with poor prognosis and

Table 3. Multivariate analysis of the association with overall and disease-free survival of Dukes B and Dukes C CRC patients in Teikyo University and Kyushu University in 2000 to 2008

	Training set ($n = 381$)			
	Multivariate analysis in Dukes B patients ($n = 151$)		Multivariate analysis in Dukes C patients ($n = 131$)	
Characteristics	Multivariate analysis for overall survival HR (95% CI)	Multivariate analysis for disease-free survival HR (95% CI)	Multivariate analysis for overall survival HR (95% CI)	Multivariate analysis for disease-free survival HR (95% CI)
PLS3 in PB	4.07 (1.50–11.57)	2.73 (1.16–6.24)	2.57 (1.42–4.63)	2.19 (1.10–4.34)
Tumor size	1.42 (0.55–3.70)	0.68 (0.28–1.56)	0.95 (0.50–1.81)	0.76 (0.35–1.63)
Lymphatic invasion	2.05 (0.62–6.30)	1.40 (0.50–3.61)	1.13 (0.62–2.08)	1.52 (0.75–3.09)
Venous invasion	0.41 (0.13–1.18)	0.50 (0.23–1.06)	1.49 (0.79–2.99)	1.32 (0.63–2.95)
Histologic type	1.05 (0.27–5.33)	0.83 (0.31–2.48)	0.60 (0.32–1.16)	0.65 (0.32–1.36)
Depth of invasion	1.27 (0.06–8.56)	0.92 (0.05–5.47)	6.94 (1.40–125.91)	2.74 (0.74–17.89)
CEA	1.10 (0.37–3.07)	1.26 (0.52–2.97)	1.33 (0.76–2.36)	1.53 (0.81–2.98)
Age	1.04 (0.99–1.09)	1.00 (0.97–1.05)	1.00 (0.98–1.03)	1.67 (0.41–7.35)
Gender	1.93 (0.73–5.70)	1.60 (0.70–3.89)	1.45 (0.79–2.71)	1.70 (0.86–3.41)
	Validation set ($n = 330$)			
	Multivariate analysis in Dukes B patients ($n = 158$)		Multivariate analysis in Dukes C patients ($n = 103$)	
Characteristics	Multivariate analysis for overall survival HR (95% CI)	Multivariate analysis for disease-free survival HR (95% CI)	Multivariate analysis for overall survival HR (95% CI)	Multivariate analysis for disease-free survival HR (95% CI)
PLS3 in PB	4.60 (1.34–16.36)	4.46 (1.73–11.62)	7.01 (2.80–19.41)	4.37 (2.15–8.94)
Tumor size	0.99 (0.30–3.07)	0.52 (0.19–1.31)	0.66 (0.25–1.63)	0.93 (0.44–1.90)
Lymphatic invasion	1.69 (0.52–5.41)	1.78 (0.67–4.56)	1.15 (0.40–3.65)	1.24 (0.57–2.78)
Venous invasion	3.51 (0.87–23.57)	1.36 (0.53–3.81)	4.18 (0.95–29.90)	1.40 (0.56–3.89)
Histologic type	0.70 (0.21–2.74)	0.84 (0.34–2.17)	0.91 (0.34–2.41)	1.15 (0.55–2.40)
Depth of invasion	1.73 (0.04–9.50)	1.26 (0.06–6.69)	3.20 (0.52–63.93)	2.97 (0.60–53.95)
CEA	0.82 (0.24–2.62)	1.42 (0.57–3.60)	1.95 (0.78–5.17)	1.17 (0.58–2.32)
Age	1.63 (0.04–8.51)	1.02 (0.97–1.06)	2.46 (0.21–14.27)	0.82 (0.13–5.68)
Gender	1.54 (0.49–5.96)	1.97 (0.79–5.59)	0.99 (0.38–2.80)	1.72 (0.82–3.83)

NOTE: Depth of invasion, muscularis propria invasion, positive or negative; lymphatic invasion, positive or negative; lymph node metastasis, positive or negative; venous invasion, positive or negative; histologic type, well differentiated or moderately and poorly differentiated and mucinous types; CEA, CEA expression, positive or negative in peripheral blood.
Abbreviations: PB, peripheral blood; CEA, carcinoembryonic antigen.

recurrence. *PLS3* is a specific, novel marker for the detection of CTCs, including EMT-induced tumor cells with a putative stem cell phenotype. Therefore, *PLS3* potentially overcomes the limitations of current epithelial markers, such as cytokeratins or EpCAM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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