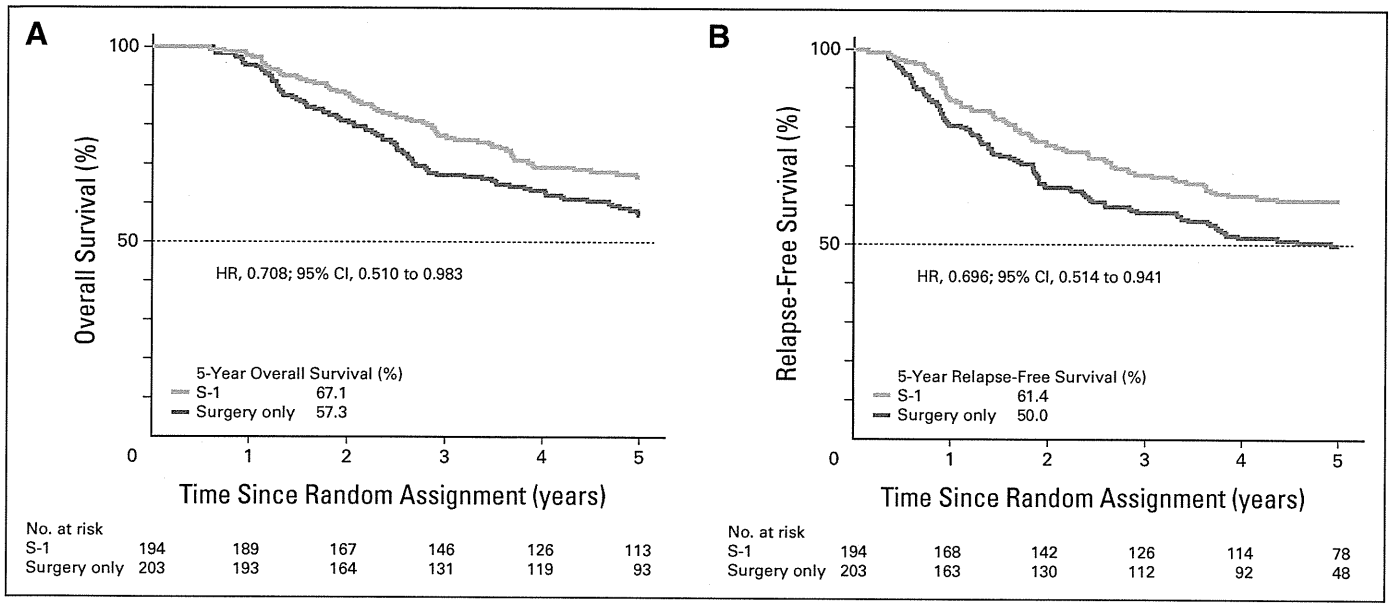

Fig 5. Kaplan-Meier estimates of (A) overall survival and (B) relapse-free survival for eligible patients with stage IIIA gastric cancer. HR, hazard ratio.

0.514 to 0.941; Fig 5B). As for stage IIIB disease, we enrolled 90 patients in the S-1 group and 85 in the surgery-only group; the 5-year OS rates were 50.2% (95% CI, 39.5% to 61.0%) in the S-1 group and 44.1% (95% CI, 33.1% to 55.0%) in the surgery-alone group, with an HR of 0.791 (95% CI, 0.520 to 1.205; Fig. 6A). Their 5-year RFS rates were 37.6% (95% CI, 27.0% to 48.2%) in the S-1 group and 34.4% (95% CI, 24.1% to 44.7%) in the surgery-alone group, with an HR of 0.788 (95% CI, 0.539 to 1.151; Fig 6B).

Site of First Relapse

Common sites of first relapse were the peritoneum, hematogenous sites, and lymph nodes (Table 1). Rates of metastasis and relapse were consistently lower in the S-1 group than in the surgery-only group for all sites. In particular, the rates of recurrence in lymph nodes and of peritoneal relapse were markedly lower in the S-1 group.

DISCUSSION

To the best of our knowledge, the ACTS-GC study is the first large clinical trial of adjuvant chemotherapy enrolling more than 1,000 patients who underwent D2 gastrectomy for gastric cancer. The results of this follow-up study showed that 1-year treatment with S-1 improved OS and RFS at 5 years compared with surgery alone, thus reconfirming the conclusions reached on early publication of the study results after a median follow-up of 3 years.

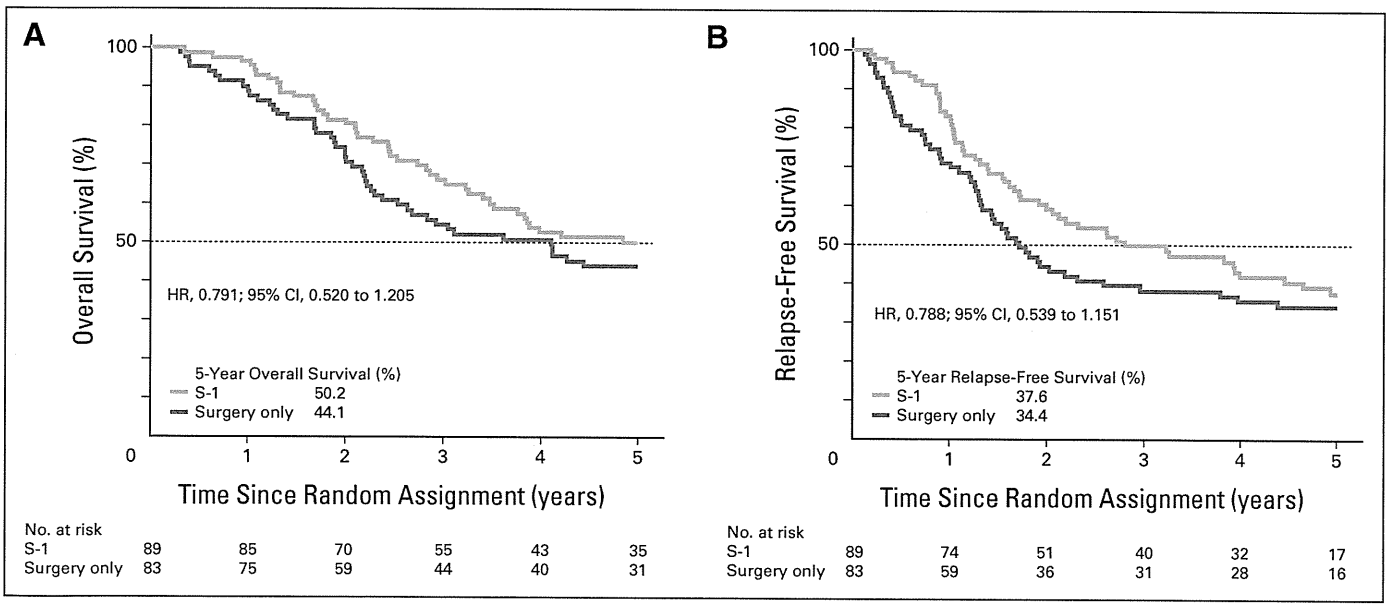

Fig 6. Kaplan-Meier estimates of (A) overall survival and (B) relapse-free survival for eligible patients with stage IIIB gastric cancer. HR, hazard ratio.

Table 1. Site of First Relapse (all randomly assigned patients)*

Site	S-1 (n = 529) No.	S-1 (n = 529) %	Surgery Only (n = 530) No.	Surgery Only (n = 530) %	HR	95% CI
Total No. of relapses	162	30.6	221	41.7	—	—
Local	11	2.1	17	3.2	0.572	0.268 to 1.221
Lymph nodes	30	5.7	54	10.2	0.505	0.323 to 0.789
Peritoneum	77	14.6	100	18.9	0.687	0.511 to 0.925
Hematogenous	61	11.5	71	13.4	0.784	0.557 to 1.105

Abbreviation: HR, hazard ratio.
*Some patients had a first relapse at more than one site.

Our present results confirmed that postoperative adjuvant chemotherapy with S-1 alone reduced the risk of death by 33.1%, thereby demonstrating that effectiveness was maintained since the previous analysis. This reduction in the risk of mortality is comparable with that obtained with combined regimens for adjuvant chemotherapy in the Medical Research Council Adjuvant Gastric Infusional Chemotherapy (MAGIC) trial[14] and the Intergroup 0116 (INT-0116) trial.[15]

Whether the results of this study can be extrapolated to countries outside East Asia remains uncertain because of possible differences in pharmacokinetics of S-1 between whites and East Asians. If S-1 is used as adjuvant chemotherapy in whites, the dose should be carefully adjusted. A second reason is that all patients in this study underwent D2 gastrectomy although more limited surgery (D0/1) is commonly performed in the United States and some parts of Europe. In the surgery-only group, OS at 5 years was 61.1%, which was much better than that of patients undergoing D2 gastrectomy in Europe (33%) in a Dutch trial.[16] One of the reasons for this large difference may be the high level and widespread use of diagnostic technology in Japan, potentially leading to stage migration between Japan and Western countries.[17] Another important reason might be the high quality of D2 gastrectomy in Japan, whereas D0 or D1 gastrectomy remains the standard procedure in the United States and was the standard in Europe until recently. Although a Dutch trial comparing D1 with D2 gastrectomy reported negative results,[16,18] a 15-year follow-up study showed that the rate of mortality from gastric cancer was significantly lower in the D2 gastrectomy group.[19] Thus, the most recent European Society for Medical Oncology (ESMO) clinical practice guidelines recommend D2 gastrectomy as the standard procedure for curable advanced gastric cancer.[20]

The primary end point of this study was 5-year OS, although that of an ongoing adjuvant chemotherapy study in Korea and China is 3-year disease-free survival. The latter is designed to evaluate the efficacy of postoperative adjuvant chemotherapy with capecitabine and oxaliplatin compared with surgery alone. To justify the use of RFS or disease-free survival as the primary end point for adjuvant chemotherapy after curative resection of gastric cancer, more evidence is needed, but the results of this study may strongly suggest that RFS can be used as the primary end point of such studies. (In this follow-up analysis, the 3-year RFS rates were 72.4% and 61.1%, and the 5-year OS rates were 71.7% and 61.1% in the S-1 group and surgery-only group, respectively.)

To compare our results with those of other foreign studies, we also report the stage-specific 3- and 5-year OS and RFS according to the International Union Against Cancer (UICC) TNM Classification of Malignant Tumours, Sixth Edition. Three-year OS rates according to UICC staging in the S-1 and surgery-only groups were 91.1% and 80.9% (stage II), 77.8% and 68.3% (stage IIIA), 66.6% and 56.8% (stage IIIB), and 59.1% and 45.7% (stage IV). Three-year RFS rates were 84.3% and 73.5% (stage II), 69.1% and 56.7% (stage IIIA), 44.8% and 28.9% (stage IIIB), and 46.0% and 37.1% (stage IV). Five-year OS rates were 83.4% and 70.8% (stage II), 68.9% and 56.2% (stage IIIA), 43.7% and 40.1% (stage IIIB), and 45.1% and 42.7% (stage IV). Five-year RFS rates were 77.9% and 65.4% (stage II), 64.3% and 48.7% (stage IIIA), 35.9% and 28.9% (stage IIIB), and 26.8% and 25.0% (stage IV).

The approach for adjuvant chemotherapy differs among East Asian countries, including Japan, in which D2 gastrectomy has long been the standard procedure, and Western countries, in which D0 or D1 gastrectomy used to be or currently is standard. As Cunningham and Chua[21] stated, "surgery alone" is no longer standard treatment anywhere in the world for advanced gastric cancer. Some type of adjuvant chemotherapy, including the use of radiotherapy after D0/1 resection, can thus be considered standard treatment at present.

A meta-analysis by the Global Advanced/Adjuvant Stomach Tumor Research International Collaboration (GASTRIC) group[7] showed that some form of postoperative chemotherapy is associated with a higher survival rate than surgery alone; moreover, the use of monotherapy for postoperative adjuvant treatment resulted in good outcomes. The ACTS-GC trial demonstrated that S-1 monotherapy improved OS and RFS. In patients with early-stage (II and IIIA) tumors, the benefits of treatment with S-1 were considerable. However, the 5-year OS rate in patients with stage IIIB disease was 50.2% in the S-1 group and 44.1% in the surgery-only group, suggesting that there remains some room for improvement. Future studies should evaluate the effectiveness of intensive preoperative and/or postoperative chemotherapy with multiple agents in patients at high risk for relapse.

The results of the S-1 plus cisplatin versus S-1 in randomized controlled trial in the treatment for stomach cancer (SPIRITS) trial,[22] demonstrating that S-1 plus cisplatin is superior to S-1 alone with respect to survival in patients with unresectable or recurrent gastric cancer, and the V325 study [a randomized, multinational phase II/III trial of patients with untreated advanced gastric cancer],[23,24] showing that the addition of docetaxel to cisplatin plus fluorouracil prolongs survival, indicated that S-1 plus cisplatin and S-1 plus docetaxel are candidate regimens for postoperative adjuvant chemotherapy. These regimens were confirmed to be feasible in a postoperative setting,[25,26] and further studies should be performed to examine whether such regimens are superior to S-1 alone.

The Japan Clinical Oncology Group (JCOG) is now performing the JCOG 0501 study to compare S-1 plus cisplatin as neoadjuvant chemotherapy with surgery followed by S-1 monotherapy in patients with clinically resectable Borrmann type 4 (linitis plastica) and large type 3 gastric cancer. This trial is expected to be a landmark study, determining the future direction for preoperative chemotherapy in Japan.

The use of molecular targeted agents for gastric cancer has been studied extensively. In the Trastuzumab in Combination with Chemotherapy Versus Chemotherapy Alone for Treatment of HER2-Positive Advanced Gastric or Gastro-Esophageal Junction Cancer (ToGA) study, trastuzumab combined with cisplatin and either fluorouracil or capecitabine significantly prolonged OS in patients with HER2-positive gastric cancer.[27] The effectiveness of adjuvant chemotherapy with molecular targeted agents such as trastuzumab also needs to be assessed in patients with HER2-positive gastric cancer.

In conclusion, this 5-year follow-up study confirmed that adjuvant chemotherapy with S-1 given for 1 year after surgery improved

OS and RFS at 5 years in patients with stage II or III gastric cancer who underwent D2 gastrectomy. Postoperative chemotherapy with S-1 can be recommended for patients with stage II or III gastric cancer who undergo D2 gastrectomy, at least in Asian populations.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Final approval of manuscript: All authors

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RESEARCH ARTICLE Open Access

Therapeutic potential of PRL-3 targeting and clinical significance of *PRL-3* genomic amplification in gastric cancer

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Abstract

Background: Phosphatase of regenerating liver-3 (PRL-3) has deserved attention as a crucial molecule in the multiple steps of metastasis. In the present study, we examined the mechanisms regulating PRL-3 expression, and assessed the clinical potential of PRL-3-targeted therapy in gastric cancer.

Methods: PRL-3 genomic amplification was analyzed using quantitative-polymerase chain reaction and/or fluorescence in situ hybridization in 77 primary gastric tumors. The anticancer activity of PRL-3 inhibitor (1-4-bromo-2-benzylidene rhodanine) treatment was evaluated against cancer cells with different genetic and expression status.

Results: PRL-3 genomic amplification was closely concordant with high level of its protein expression in cell lines, and was found in 20% (8/40) among human primary tumors with its expression, which were all stage III/IV disease (40%, 8/20), but in none (0/37) among those without expression. Additionally, PRL-3 genomic amplification was associated with metastatic lymph node status, leading to advanced stage and thereby poor outcomes in patients with lymph node metastasis ($P = 0.021$). PRL-3 small interfering RNA robustly repressed metastatic properties, including cell proliferation, invasion, and anchorage-independent colony formation. Although neither PRL-3 genomic amplification nor expression level was responsible for the sensitivity to PRL-3 inhibitor treatment, the inhibitor showed dose-dependent anticancer efficacy, and remarkably induced apoptosis on all the tested cell lines with PRL-3 expression.

Conclusions: We have for the first time, demonstrated that PRL-3 genomic amplification is one of the predominant mechanisms inducing its expression, especially in more advanced stage, and that PRL-3-targeted therapy may have a great potential against gastric cancer with its expression.

Keywords: PRL-3 gastric cancer, genomic amplification, targeted therapy, lymph node

Background

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death worldwide [1]. Recent improvements in diagnostic tools and methods have facilitated detection of early GC and thereby excellent long-term survival. However, patients with advanced disease at the time of diagnosis remain poor outcomes. Metastasis is a multistep process, involving local invasion, dissemination, and re-establishment into distant organs, and is the major determinant of the mortality [2]. Therefore, a better understanding of metastasis may open the way to a host of innovative therapeutic strategies in GC.

The protein tyrosine phosphatases (PTPs) form a large family of enzymes that serve as key regulatory components in signal transduction pathways [3]. The phosphatases of regenerating liver (PRL-1, -2, and -3), belonging to a small class of PTP superfamily, have a unique COOH-terminal prenylation motif, which critically affects their cellular localization and function [4]. PRL-3 was firstly identified to be specifically over-expressed in

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liver metastases derived from colorectal cancer [5], and subsequently its overexpression was documented in various tumor types, including GC [6]. PRL-3 can promote cancer invasion, migration, growth, and angiogenesis, through either dephosphorylation that is catalyzed by catalytic domain or localization to plasma membrane directed by COOH-terminal prenylation motif [7-9]. Thus, PRL-3 has deserved attention as a crucial molecule in the multiple steps of metastasis and therefore as a new therapeutic target. On the other hand, the mechanisms inducing PRL-3 expression are not fully clarified. Amplification of genomic regions containing oncogenes is the major mechanism of its consequent overexpression and the cancer development, and therefore has importance for targeted therapies [10]. *PRL-3* gene amplification partially accounts for the overexpression in colorectal cancer and esophageal cancer [5,11]. However, the relationship between genomic amplification and GC remains elusive in the both mechanistic and therapeutic points of view. In the present study, we examined the characteristics of *PRL-3* genomic amplification in GC, and further assessed the clinical potential of PRL-3-targeted therapy.

Methods

Cell lines and Tissue Samples

The GC cell line MKN7 was kindly provided from the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). Seven other GC cell lines (GCIY, AZ521, KatoIII, SH10, H111, MKN74, and NUGC4) were purchased from RIKEN BioResource Center (Ibaraki, Japan). These cell lines cover the two main types of GC [12], intestinal type (MKN7, MKN74, AZ521, and H111 cells) and diffuse type (GCIY, KatoIII, SH10, and NUGC cells) [13-15]. MKN7, NUGC4, and AZ521 cells were established from lymph node metastasis (LNM), and MKN74 cells were from liver metastasis. KATOIII and GCIY cells were established from metastatic pleural effusion and ascites, respectively. H111 and SH10 cells were established from the xenotransplantation. Normal skeletal muscle C2C12 cells were purchased from DS Pharma Biomedical Co., Ltd (Osaka, Japan). AZ521 and C2C12 cells were grown in DMEM medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). The other cells were grown in RPMI1640 medium (GIBCO) supplemented with 10% FBS. 1-4-bromo-2-benzylidene rhodanine was purchased from Calbiochem Corp (San Diego, CA), which was identified as a PRL-3 inhibitor through high throughput screening using chemical library of Korea Chemical Bank, and inhibited PRL-3 phosphatase activity [16]. Indeed, phosphorylation of KRT8, PRL-3-interacting protein, induced by catalytically inactive mutant of PRL-3, but not by wild type, was confirmed by PRL-3 inhibitor treatment in a dose-dependent manner [17]. Moreover, anticancer efficacy of PRL-3 inhibitor treatment also showed to be similar to that of siRNA treatment in esophageal cancer or colorectal cancer [11,17].

Out of 173 formalin-fixed, paraffin-embedded, tissue samples series where we previously assessed PRL-3 expression status using immunohistochemical staining (IHC) in GC [6], 77 matched pairs of primary tumor tissues and the corresponding normal mucosa tissues were randomly selected from patients with differential stages according to the 13th edition of the Japanese Classification of Gastric Carcinoma (JCGC) [18]; 40 pairs with positive PRL-3 expression (10 patients in Stage I, 10 in II, 10 in III, and 10 in IV) and 37 pairs with negative expression (10 patients in stage I, 10 in II, 9 in III, and 8 in IV). All patients underwent gastrectomy according to the gastric cancer treatment guidelines in Japan [19], and histopathologic examinations were done according to the JCGC. The 6th edition of the International Union Against Cancer (UICC)/TNM classification was also used [20]. Table 1 depicts the detailed information on 77 patients. All tissue samples were collected at the Kitasato University Hospital, and informed consent was obtained from all patients. The present study was approved by the Ethics Committee of the Kitasato University.

Fluorescence in situ hybridization analysis

Fluorescence in situ hybridization (FISH) analysis was performed, as described previously [11]. *PRL-3* is located on chromosome 8q24.3 (GenBank accession number NT 000008.9), and the chromosome 8 centromeric probe was used to estimate the copy number. Because *PRL-3* FISH scoring algorithms had not been standardized, the assessment was based on the criteria of *HER2* [21]. For each sample, at least 60 cancer cells were scored. Positive *PRL-3* genomic amplification was defined as a ratio of *PRL-3* to chromosome 8 centromere more than 2.2, and negative was the ratio of less than 1.8. If the ratio of *PRL-3* to chromosome 8 centromere was 1.8 to 2.2, additional cells were counted, and the ratio of more than 2.0 was finally considered as positive [21]. Polysomy was defined as the mean chromosome 8 centromeric signals more than 3.0 per nucleus [22].

Quantitative-genomic PCR

Tissue sections from tumor and the corresponding normal mucosa, obtained at least 5 cm from the tumor edge, were sharply dissected on hematoxylin and eosin-stained slides, and genomic DNA was subsequently extracted using of a QIAamp DNA FFPE Kit (QIAGEN Sciences, Hilden). Quantitative-genomic polymerase chain reaction (Q-PCR) was performed to quantify

Table 1 Correlation between PRL-3 gene amplification and clinicopathological variables in 77 patients with gastric cancer

Variables	Total number	PRL-3 gene amplification				*p* value
		Negativity		Positivity		
		Number	(%)	Number	(%)	
PRL-3 expression						0.006
Negativity	37	37	(100)	0	(0)	
Positivity	40	32	(80)	8	(20)	
Age (years)						0.726
<60	34	30	(88)	4	(12)	
≥60	43	39	(91)	4	(9)	
Gender						0.710
Male	51	45	(88)	6	(12)	
Female	26	24	(92)	2	(8)	
Lymphatic permeation						0.343
Absence	15	15	(100)	0	(0)	
Presence	62	54	(87)	8	(13)	
Vascular permeation						0.263
Absence	25	24	(96)	1	(4)	
Presence	52	45	(87)	7	(13)	
Differentiation						0.134
Well and moderate	31	30	(97)	1	(3)	
Poor	46	39	(85)	7	(15)	
Depth of invasion						0.006*
T1 (m and sm)	15	15	(100)	0	(0)	
T2 (mp and ss)	35	33	(94)	2	(6)	
T3 (se)	19	16	(84)	3	(16)	
T4 (si)	8	5	(63)	3	(38)	
Lymph node metastasis						0.022
Absence	29	29	(100)	0	(0)	
Presence	48	40	(83)	8	(17)	
JCGC lymph node status[†]						0.004*
N0	29	29	(100)	0	(0)	
N1	21	20	(95)	1	(5)	
N2	20	14	(70)	6	(30)	
N3 and distant lymph nodes	7	6	(86)	1	(14)	
UICC lymph node status[‡]						0.002*
N0	29	29	(100)	0	(0)	
N1	18	17	(94)	1	(6)	
N2	16	13	(81)	3	(19)	
N3 and distant lymph nodes	14	10	(71)	4	(29)	
JCGC stage						0.005*
I (IA and IB)	20	20	(100)	0	(0)	
II	20	20	(100)	0	(0)	
III (IIIA and IIIB)	19	15	(79)	4	(21)	
IV	18	14	(78)	4	(22)	
UICC stage						0.003*
I (IA and IB)	21	21	(100)	0	(0)	
II	20	20	(100)	0	(0)	
III (IIIA and IIIB)	16	13	(81)	3	(19)	
IV	20	15	(75)	5	(25)	

PRL-3 gene copy numbers using iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA) in triplicate on the iCycler iQ™ Real-Time PCR Detection system (Bio-Rad). To normalize *PRL-3* gene copy number per cell, ADAM metallopeptidase domain 2 (*ADAM2*, NT 923907.1), located on chromosome 8p11.2, was used as an endogenous reference because that gene amplification is defined as a copy number increase of a restricted region of a chromosome arm [10]. ΔCt values were calculated as Ct (*PRL-3*)-Ct (*ADAM2*) for each sample. Relative copy number was determined as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t$ (tumor)-ΔC_t (corresponding normal) [23]. The increases of more than 2-fold relative to the corresponding normal were considered as genomic amplification. Additional file 1 depicts detailed PCR condition and sequences of primer and probe used in the present study.

Western blotting

Whole cell lysates were extracted in RIPA buffer (Pierce, Rockford, IL) supplemented with 10 μL/mL Halt™ Protease Inhibitor Cocktail Kit (Pierce) and Halt™ Phosphatase Inhibitor Cocktail Kit (Pierce), and the protein were separated on NuPAGE® 4-12% Bis-Tris Gel (Invitrogen) according to the manufacturer's protocol. Both detection and quantification of the specific proteins were performed using ATTO Light Capture (ATTO Corporation, Tokyo, Japan). Two colorectal cancer cell lines DLD-1 and SW480 cells (RIKEN BioResource) were used as the low and high expression controls, respectively, as described previously [11].

PRL-3 mouse monoclonal antibody (R&D Systems, Minneapolis, MN) and β-actin mouse monoclonal antibody (Sigma, St. Louis, MO) were used as described previously [11].

PRL-3 small interfering RNA transfection

Cells were transfected with 1 μmol/L Accell SMARTpool, siRNA-PRL-3 (Thermo Fisher Scientific, Lafayette, CO) mixed with Accell siRNA Delivery Media (Thermo Fisher Scientific) according to the Thermo Scientific Dharmacon® Accell™ siRNA Delivery Protocol [24]. The Accell Non-targeting Pool (siRNA-ctr) and Accell siRNA Delivery Media alone were used as a control for non-sequence-specific effects and as a mock-treatment, respectively.

Anchorage-independent colony formation assay

Anchorage-independent cell growth was analyzed by plating 0.36% top agarose (Bacto™ Agar, Becton, Dickinson and Company, Franklin Lakes, NJ) containing 1×10^5 cells on a surface of 0.72% bottom agarose in 6-well plates [11]. Cells were fed weekly by overlying fresh soft-agar solution, and colonies were photographed after 2 weeks of incubation. The 50% effective concentration (EC_{50}) value of PRL-3 inhibitor treatment was calculated based on the measurement of colony count.

Proliferation assay and invasion assay

The proliferation assay was performed using Premix WST-1 Cell Proliferation Assay System (Takara Bio, Tokyo). Cells (2×10^3) were seeded in 96-well, and the proliferative activity was measured by absorbance at 450 nm on designated sampling days. The sensitivity to PRL-3 inhibitor on antiproliferation was determined using the 50% inhibitory concentration (IC_{50}) value after treatment for 72 hours.

The invasion assay was performed in the 24-well BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences Discovery Labware, Bedford, MA). Cells that had invaded through the membrane were counted in four separated fields per well. Both experiments were done in triplicate.

Apoptosis Assays

Apoptosis assays were performed using Guava PCA System (Guava Technologies, Inc., Hayward, CA). Cells (2×10^5) were treated with the PRL-3 inhibitor at the indicated concentration in medium supplemental with 1.0% FBS for 72 hours, then stained with Annexin V and 7-AAD (Guava Nexin Reagent). The experiment was done in triplicate and analyzed using CytoSoft 2.1.5 software (Guava Technologies).

Statistical Analysis

Fisher's exact test, or the Mann-Whitney *U*-test was used to statistically analyze the relationship between *PRL-3* gene amplification and clinicopathological variables. One-way analysis of variance (ANOVA) with post-hoc test was used to compare between three groups for siRNA treatment (siRNA-PRL-3, siRNA-ctr, and mock). Student *t* test was used to evaluate therapeutic effect for the individual concentrations of PRL-3 inhibitor, compared with 0 μmol/L of PRL-3 inhibitor. The Kaplan-Meier method was used to estimate cumulative survival rates, and differences in survival rates were assessed with the use of the log-rank test. All deaths of patients were cancer-related, and disease specific survival (DSS) was measured from the date of surgery to the date of death or the last follow-up. $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were conducted with JMP 7.0 software (SAS Institute, Cary, NC).

Results

PRL-3 expression and genomic amplification in gastric cancer cell lines

Initially, PRL-3 expression status was evaluated using western blotting in 8 GC cell lines (Figure 1A). PRL-3

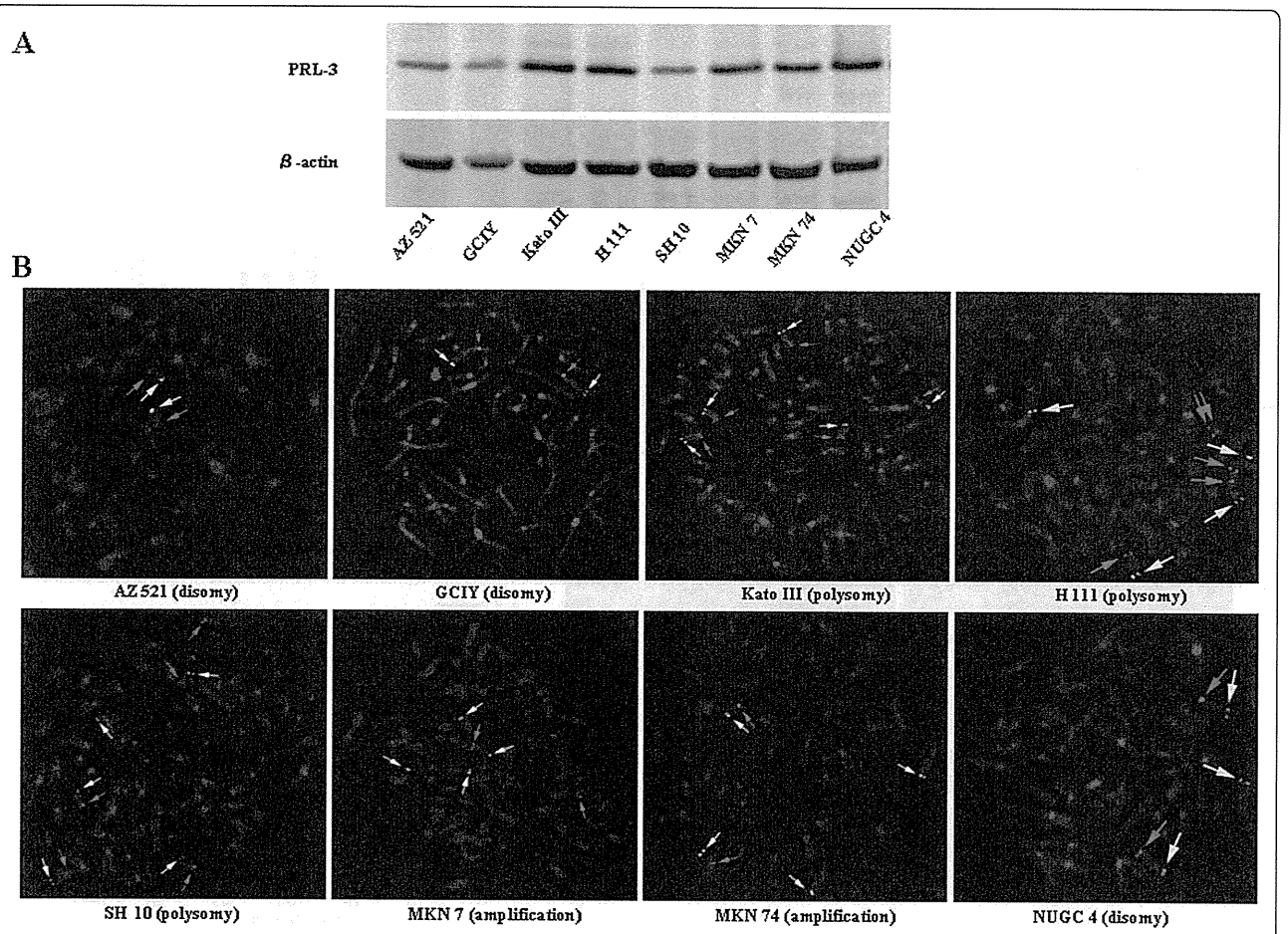

Figure 1 Expression and genetic status of PRL-3 in 8GC cell lines. (A) Expression level of PRL-3 by western blotting. (B) FISH analysis on metaphase of *PRL-3* gene (green). The chromosome 8 centromeric probe (orange) was used to estimate the copy number.

expression was observed at a detectable level in all the cell lines, among which 5 cell lines (KatoIII, H111, MKN7, MKN74, and NUGC4 cells) and 3 cell lines (GCIY, AZ521, and SH10 cells) exhibited high and relatively low expression, respectively. Subsequently, FISH analysis was performed to examine whether PRL-3 expression was caused through its genomic amplification (Figure 1B). Genomic amplification was obviously positive in 2 cell lines (MKN7 and MKN74 cells) and negative in 6 cell lines. 3 of the six were dysomic (AZ521, GCIY, and NUGC4 cells), and three were polysomic (KatoIII, SH10, and H111 cells). *PRL-3* genomic amplification frequently occurred in the different regions from chromosome 8, so-called distributed insertions, on metaphase [10], and was concordant with its high expression.

Characteristic of *PRL-3* genomic amplification in human primary gastric cancers

In our previous study, PRL-3 expression was detected in 95 (55%) out of 173 primary GCs by IHC [6]. To explore the link between PRL-3 expression and its genomic amplification, Q-PCR was performed for both the 40 tumors with positive PRL-3 expression and 37 tumors with negative expression, which were randomly selected from differential stages in the 173 primary tumors. All the primary tumors without PRL-3 expression were not amplified, whereas 8 (20%) out of the 40 primary tumors with PRL-3 expression were amplified (Figure 2A). FISH analyses also confirmed obvious genomic amplification as the cancer-specific alteration (Figure 2B), and exhibited at nearly homogenous pattern in both the central area and invasive area within tumor. Subsequently, the relationship with clinicopathological factors was assessed for *PRL-3* genomic amplification (Table 1), where it was significantly associated not only with its expression ($P = 0.006$), but also with depth of tumor invasion ($P = 0.006$), presence of LNM ($P = 0.022$), LNM status ($P = 0.004$ in JCGC, $P = 0.002$ in UICC), and stage ($P = 0.005$ in JCGC, $P = 0.003$ in UICC). Additionally, all the primary tumors with genomic amplification were stage III or IV disease (40%, 8/20). Moreover, the genomic amplification negatively affected

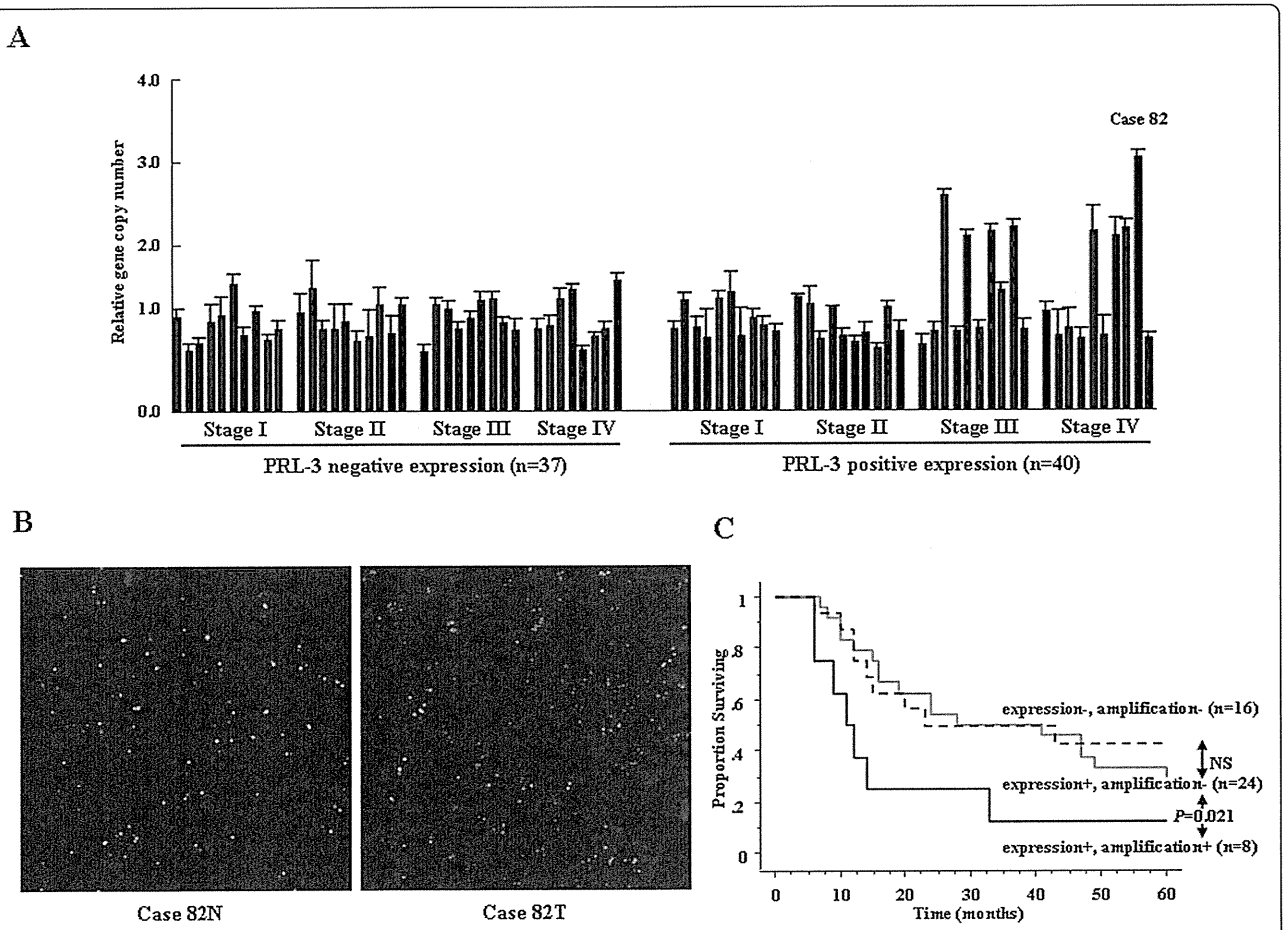

Figure 2 Frequency and prognosis of *PRL-3* genomic amplification in 77 human GC. (A) Frequency of *PRL-3* genomic amplification using Q-PCR in 77 human GC. Q-PCR was performed for both the 40 tumors with positive PRL-3 expression and 37 tumors with negative expression. To normalize *PRL-3* gene copy number per cell, *ADAM2*, located on chromosome 8p11.2, was used as an endogenous reference. ΔCt values were calculated as Ct (*PRL-3*)-Ct (*ADAM2*) for each sample. Relative copy number was determined as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t$ (tumor)-ΔC_t (corresponding normal). (B) Representative FISH analysis of *PRL-3* gene in primary tumor and corresponding normal (case 82). (C) Kaplan Meier curves of 5-year DSS according to the positivity or negativity of *PRL-3* genomic amplification in the histologically node-positive patients. Error bars, standard deviation (SD).

the outcomes of the histologically node-positive patients (P = 0.021, Figure 2C), although PRL-3 expression did not in our and other previous reports [6,25].

PRL-3 as a convergent therapeutic target

In GC, the functional roles of PRL-3, including invasion and proliferation abilities, have been documented only in SGC7901 cells [25]. To confirm these metastatic properties using 3 cell lines with different PRL-3 expression and genetic status, knock-down of endogenous PRL-3 expression was performed using siRNA transfection; AZ521 cells (low expression and disomy), H111 cells (high expression and polysomy), MKN74 cells (high expression and genomic amplification). These cell lines were transfected with siRNA-PRL-3 or siRNA-ctr, and western blotting showed the decreased level of PRL-3 protein in siRNA-PRL-3 cells, but not siRNA-ctr cells, compared with mock-treatment cells (Figure 3A). One of the important characteristic of the metastatic phenotype is supposed as the ability for cancer cells to grow under anchorage-independent conditions [26], but the involvement in PRL-3 remains unknown in GC. All siRNA-PRL-3 cells showed the significantly decreased size and number of colonies, compared to siRNA-ctr cells or mock-treatment cells (Figure 3B). Moreover, in line with previous reports for other GC cell lines [25,27], we also confirmed that siRNA-PRL-3 cells showed the significantly less proliferative activity (Figure 3C) and invasive ability (Figure 3D).

Therapeutic potential of PRL-3 inhibitor, 1-4-bromo-2-benzylidene rhodanine

To assess the therapeutic potential and examine a landmark guiding the response to PRL-3-targeted therapy, we evaluated the anticancer activity of PRL-3 inhibitor,

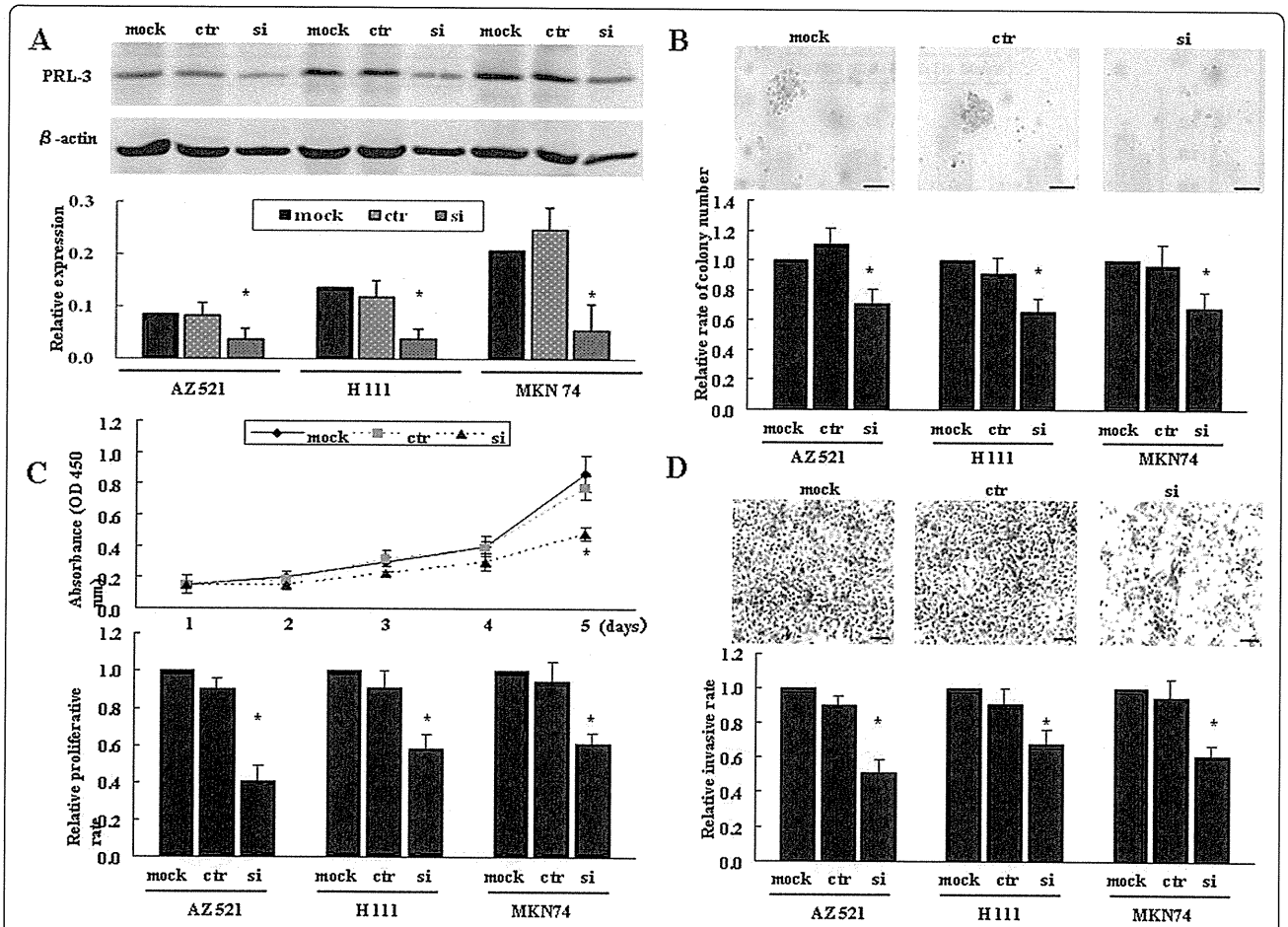

Figure 3 Inhibition of metastatic properties after transfection with siRNA in cells with expression. (A) Western blotting 72 hours after transfection. The western blotting was also quantified. mock, treatment with Accell siRNA Delivery Media alone; ctr, treatment with siRNA-ctr; si, treatment with siRNA-PRL-3. (B) Anchorage-independent colony formation assays. Representative pictures of colony formation on AZ521 cells were shown in top panel. With mock-treatment as 1.0, the relative rate of colony number was shown in bottom panel. Bars, 200 μm. (C) Proliferation assay. The proliferative activity of AZ521 cells on 1, 2, 3, 4, or 5 days after transfection (top panel) and the relative proliferative rate on 5 days after transfection (bottom panel) were shown. (D) Invasion assay. Cells were seeded on 4 days after transfection, and then incubated for 22 hours. Representative pictures of AZ521 cells (top panel) and the relative invasive rate (bottom panel) were shown. Bars, 200 μm; *, $P < 0.05$ by ANOVA with post-hoc test; error bars, SD.

cell-permeable benzylidene rhodanine compound [16], against 6 cell lines with different PRL-3 expression and genetic status; GCIY and AZ521 cells (low expression and disomy), KatoIII cells (high expression and polysomy), SH10 cells (low expression and polysomy), MKN7 and MKN74 cells (high expression and genomic amplification). Cells were treated with PRL-3 inhibitor at concentrations ranging from 0 to 50 μmol/L. PRL-3 inhibitor showed dose- and time-dependent antiproliferative efficacy on all the tested cell lines, irrespective of different PRL-3 expression level and genetic status, and the IC_{50} values of GCIY, AZ521, KatoIII, SH10, MKN7, and MKN74 cells were 26.77, 9.98, 24.26, 23.95, 22.29, and 9.45 μmol/L, respectively (Figure 4A). AZ521 and MKN74 cells were more sensitive to PRL-3 inhibitor treatment than GCIY and MKN7 cells that were categorized as the identical groups in terms of expression and genetic status, respectively. Namely, genetic or expression status was not associated with sensitivity of GC cells against the PRL-3 inhibitor. Similar efficacy was shown in anchorage-independent colony formation, and the EC_{50} values of GCIY, AZ521, SH10, and MKN74 cells were 6.99, 9.52, 13.05, 9.09 μmol/L, respectively (Figure 4B). GCIY cells exhibited more sensitive inhibition in contrast with the anti-proliferation. Additionally, this inhibitor also robustly abrogated the invasive ability of GC cells (Figure 4C). To further characterize the anticancer efficacy of PRL-3 inhibitor treatment, apoptosis assay was performed (Figure 5A). Although 1 μmol/L of the inhibitor was insufficient to induce apoptosis beyond the baseline (0 μmol/L), 10 μmol/L of the inhibitor robustly caused the drastic

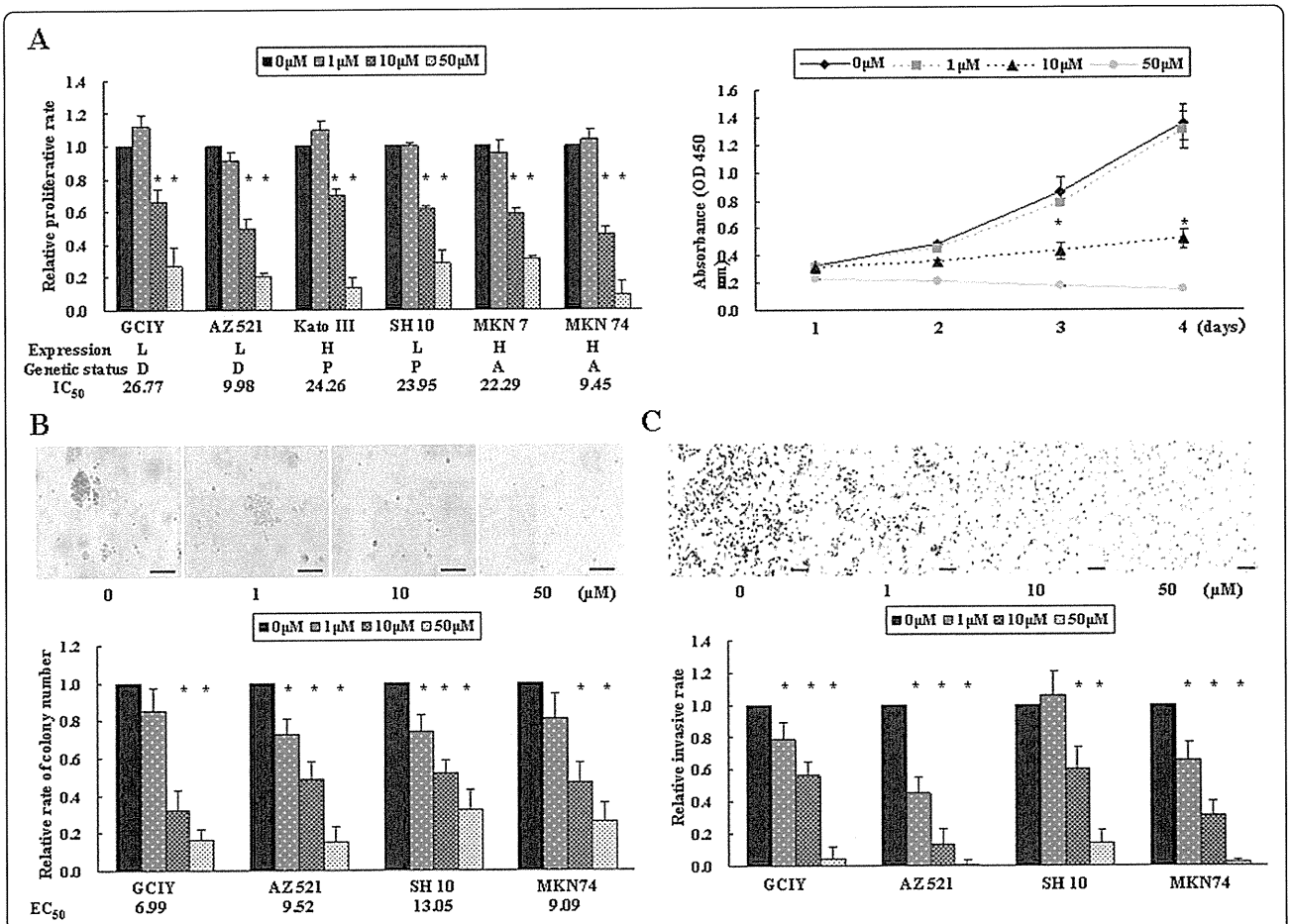

Figure 4 Anticancer activity of PRL-3 inhibitor. (A) Proliferation assay after treatment with PRL-3 inhibitor at concentrations ranging from 0 to 50 μmol/L against 6 cell lines with different PRL-3 expression and genetic status. With cells treated with chemical solution alone (0 μmol/L PRL-3 inhibitor) as 1.0, the relative proliferative rate on 5 days after treatment was shown in left panel. The proliferative activity of AZ521 cells on 1, 2, 3, or 4 days after treatment was shown in right panel. L, low expression; H, high expression; D, disomy; P, polysomy; A, amplification; IC_{50}, the 50% inhibitory concentration. (B) Anchorage-independent colony formation assays. Representative pictures of colony formation on AZ521 cells (top panel) and the relative rate of colony number (bottom panel) were shown. Bars, 200 μm; EC_{50}, the 50% effective concentration. (C) Invasion assay. Representative pictures (top panel) and the relative invasive rate (bottom panel) were shown. Bars, 200 μm; *, $P < 0.05$ by Student *t* test, compared with 0 μmol/L of PRL-3 inhibitor; error bars, SD.

apoptosis on all the tested cell lines, where there were the 3-fold and 11-fold increases beyond the baseline in GCIY and MKN74 cells, respectively. Thus, PRL-3 inhibitor repressed these metastatic properties on all the tested cell lines in dose-dependent manner, and neither expression level nor genetic status showed clear correlation with the sensitivity.

Finally, we assessed whether PRL-3 inhibitor induced cytotoxicity in normal skeletal muscle, where PRL-3 is predominantly expressed [28]. Both proliferation and apoptosis assays were performed using normal skeletal muscle C2C12 cells treated with the inhibitor, and showed that 10 μmol/L of the inhibitor failed to cause antiproliferative and apoptotic response on C2C12 in contrast with the efficacies on all the tested GC cell lines (Figure 5A and 5B).

Discussion

As LNM is considered as an important prognostic factor for GC [29], research of the causative molecules reflecting LNM is a promising avenue to improve the outcomes. The close link of LNM with PRL-3 expression, therefore, has potential as a new therapeutic target [6,25]. However, the criteria for PRL-3-targeted therapy have not been established, and it is critical to clarify the characteristics of *PRL-3* genomic amplification in the both mechanistic and therapeutic points of view, because of the major mechanism of its consequent expression and the cancer development [10]. In the present study, we offer the vital clues for the development of this therapeutic strategy against GC.

The relationship between PRL-3 expression and its genomic amplification have never been examined so far.

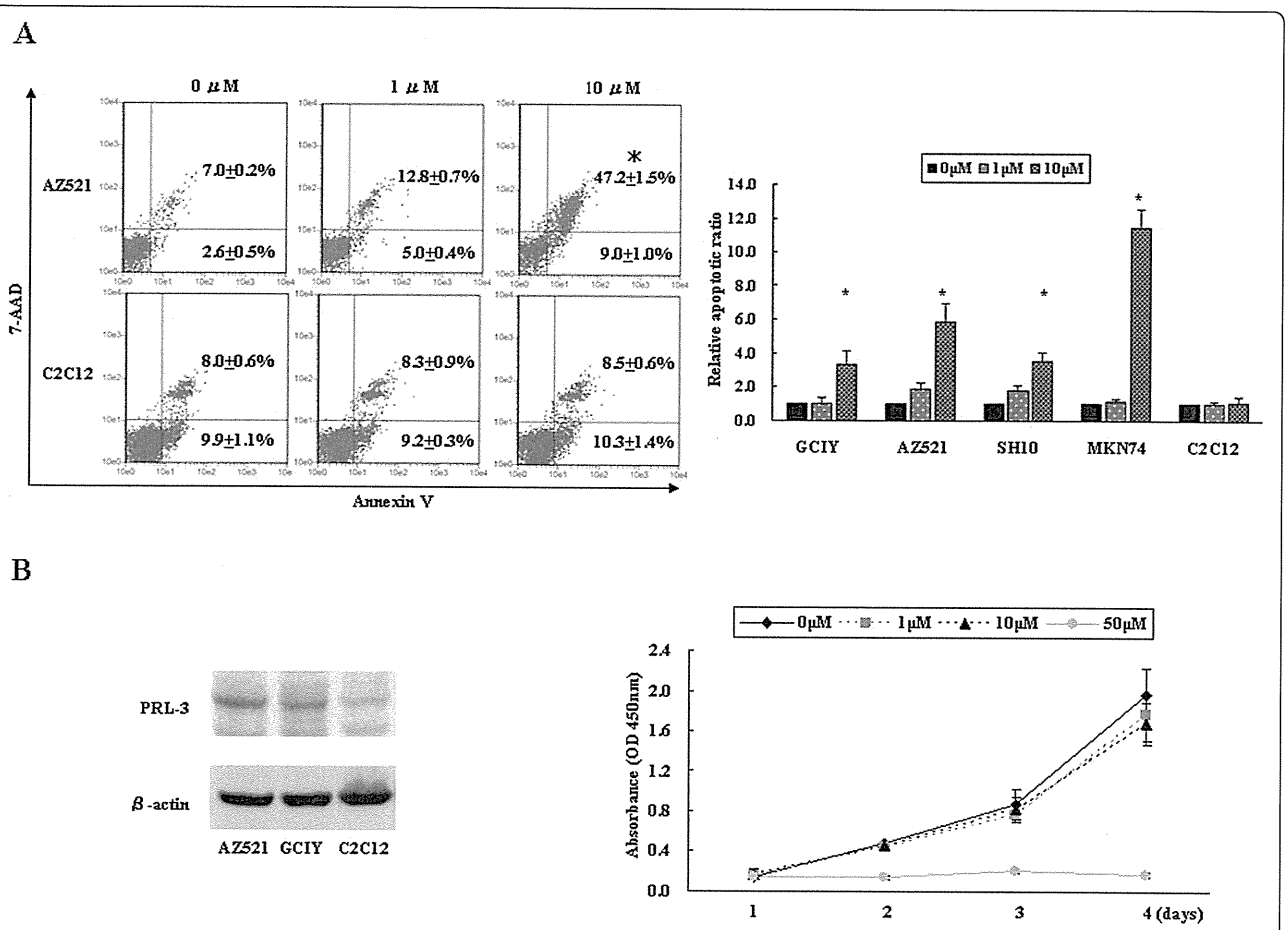

Figure 5 PRL-3 inhibitor-mediated apoptosis. (A) Apoptosis assay was performed 72 hours after treatment with PRL-3 inhibitor (0 to 10 μmol/L). Representative figures of apoptosis assay on AZ521 and C2C12 cells were shown in left panel, and the percentage and SD of early apoptosis (bottom right quadrant) and late apoptosis (top right quadrant) are shown in each panel. With cells treated with chemical solution alone (0 μmol/L PRL-3 inhibitor) as 1.0, the relative late apoptosis rate after treatment was shown in right panel. *, $P < 0.05$ by Student t test, compared with 0 μmol/L of PRL-3 inhibitor. (B) PRL-3 inhibitor treatment against normal skeletal muscle C2C12 cells. C2C12 cells exhibited lower expression level of PRL-3 than GC cells by western blotting. Proliferation assay after treatment with PRL-3 inhibitor was performed. Error bars, SD.

PRL-3 genomic amplification was concordant with its expression status in cell lines, and was found in 20% (8/40) among human primary tumors with expression, which were all stage III or IV disease (40%, 8/20), but in none (0/37) among those without expression. Additionally, *PRL-3* genomic amplification was associated with LNM status, leading to advanced stage and thereby poor outcomes in patients with LNM ($P = 0.021$). Thus, *PRL-3* genomic amplification may be the more relevant alteration for LNM, and be one of the predominant mechanisms inducing its expression in the more advanced stage. However, most tumors expressing PRL-3 were not amplified, especially in the earler stage. In mouse embryonic fibroblast cells with wild type but not $p53^{-/-}$, PRL-3 is induced in a p53-dependent manner [30]. The p53 mutation or loss of function, however, has been documented in all the GC cell lines used in the present study, except for NUGC4 cells (The TP53 Web Site, http://p53.free.fr/), indicating that there is other mechanism independently of p53 pathway. PRL-3 expression was reported to be regulated at transcriptional level by mitogenic cytokines, such as IL-6, IL-21, HGF or IGF-1 in myeloma cell lines [24], or as TGF-β in colon cancer cell lines [31]. Recently, PolyC-RNA-binding protein 1 (PCBP1) has been identified as a translational regulator of PRL-3 [32]. The alternative mechanisms at transcriptional or translational level may be involved to regulate PRL-3 expression.

We also confirmed that siRNA-mediated PRL-3 knockdown significantly repressed cell proliferation and invasion in line with previous reports for other GC cell lines [25,27], and furthermore for the first time revealed the reduced effect of colony formation under anchorage-independent conditions, supporting that PRL-3

may be attractive therapeutic target against GC. The success of molecular-targeted therapy depends on the identification of a landmark to select patients with more benefit from the therapy, such as activating mutation or gene amplification of EGFR in non-small cell lung cancer [33], and overexpression or gene amplification of HER2 in breast cancer [34]. Thus, genetic alteration or expression status is possible to be a landmark for molecular-targeted therapy, and it is indispensable to evaluate the anticancer activity of PRL-3 inhibitor treatment against cancer cells with different genetic and expression status. Although neither *PRL-3* genomic amplification nor expression level was responsible for the sensitivity to PRL-3 inhibitor treatment, the inhibitor exhibited dose-dependent efficacy on all the tested cell lines with PRL-3 expression, and remarkably induced apoptosis in line with a previous report [35]. PRL-3 is not expressed in human adult stomach, and its expression is cancer-specific event [6,7]. Collectively, the presence of PRL-3 expression, but not expression level, may be sufficient to promote metastatic properties through activation of downstream signaling pathways, and the effective inhibition seems to have important implication for the success of this treatment. Combined with our previous findings demonstrating the high frequency of PRL-3 expression (55%, 95/173) [6], PRL-3-targeted therapy may be applicable for most patients with GC. The different sensitivity against PRL-3 targeting as shown in the present study may imply the additional alterations attenuating the dependence of PRL-3 signaling networks on cancer cells. Therefore, identification of molecules leading to the different sensitivity would shed light on the development of more sophisticated strategy.

Normal tissues with PRL-3 expression may be susceptible to adverse effects from the targeted therapy, especially in normal skeletal muscle and heart [28]. Interestingly, PRL-3 inhibitor treatment with the concentration of 10 μmol/L significantly repressed proliferation through apoptosis induction on all the tested GC cell lines, whereas did not on normal skeletal muscle C2C12 cells, implying that this concentration may act as an optimal dose of anticancer activity without severe effects against muscle cells, and normal cells may have a better apoptotic protective mechanism, even though PRL-3 is constitutively expressed [35]. As C2C12 cells might not be the best control because of relatively weak expression, further research will be necessary to validate our findings.

Conclusions

We have for the first time demonstrated that *PRL-3* genomic amplification is one of the predominant mechanisms inducing its expression, especially in more advanced stage, and that PRL-3-targeted therapy may have a great potential against gastric cancer with its expression.

List of Abbreviations used

PRL-3: Phosphatase of regenerating liver-3; GC: gastric cancer; PTP: protein tyrosine phosphatase; JCGC: Japanese Classification of Gastric Carcinoma; UICC: the Union Internationale Contre Le Cancer; FISH: fluorescence in situ hybridization; Q-PCR: quantitative-genomic polymerase chain reaction; EC_{50}: 50% effective concentration; IC_{50}: 50% inhibitory concentration; ANOVA: analysis of variance; DSS: disease specific survival.

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Authors' contributions

AO conceived of the study, performed the study, drafted the manuscript and participated in coordination. KY participated in coordination and assisted in editing of manuscript. SK, SS, NK, MW, HK, and KN helped in the collection and analysis of clinical data. MW participated in coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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ORIGINAL ARTICLE

The value of pleural lavage cytology examined during surgery for primary lung cancer

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Abstract

OBJECTIVES: The pleural invasion (PL) score is a useful prognostic indicator in lung cancer. However, in many cases, the cancer may exfoliate itself into the pleural cavity and may progress to a malignant pleural effusion without invading the parietal pleura. This stage is not currently evaluated, but it is detectable by means of the pleural lavage cytology (PLC). However, PLC's contribution to TNM staging has not yet been clarified. The purpose of this investigation was to demonstrate the usefulness of PLC in the precise staging of patients with such an occult pleural dissemination.

METHODS: A total of 3231 patients who were included in a multi-institutional database were studied retrospectively. PLC was performed by washing the thoracic cavity with a small amount of physiological saline immediately after opening the thoracic cavity during lung cancer surgery.

RESULTS: The incidence of positive PLC findings was 4.58%. In comparison with the negative group, the survival curves were significantly worse ($P < 0.001$) and the incidence of recurrence with pleuritis carcinomatosa was significantly higher ($P < 0.001$). According to the subset analysis, the survival difference was prominent in earlier stage groups and lower PL score groups. The positive findings were confirmed to be a significantly poor prognostic indicator ($P = 0.016$) by multivariate analysis using the Cox proportional hazard model (Cox analysis). However, integration of the positive findings with the PL score was attempted for the convenience of TNM staging. To find the accurate PL score for positive PLC findings, the Cox analysis was re-estimated using the PL score upgraded stepwise. The most reliable model with the highest score for the likelihood ratio χ^2 statistic was obtained by scoring positive findings as PL3. So, it was considered to be the most reliable conversion.

CONCLUSIONS: Examining PLC in clinical practice is useful for detecting occult pleural dissemination before the appearance of a malignant pleural effusion. Evidence of positive findings should be treated as supplemental information to the precise diagnosis of TNM staging. Scoring positive PLC findings as PL3 (=T3) was appropriate.

Keywords: Pleural lavage cytology • Cytological examination of pleural lavage fluid • Occult pleural dissemination • TNM staging lung cancer • Non-small-cell lung cancer

INTRODUCTION

The peripheral lung cancer tends to grow by invading the visceral pleura and then progressing to the parietal pleura. This progression is expressed by a pleural invasion (PL) score from PL0 to PL3, which is considered to be useful for predicting prognosis by providing supplemental information to TNM staging [1]. However, it is evident that lung cancer may progress via another route. After reaching the surface of the visceral pleura, cancer cells may exfoliate themselves into the pleural cavity and potentially progress to a malignant pleural effusion. Although this type of progression is not currently considered for staging purposes, it is detectable by the cytological examination of the pleural cavity, such as via pleural lavage cytology (PLC). Several reports have suggested that PLC findings obtained during surgery are an important prognostic indicator [2-14]. However, PLC's contribution to TNM staging has not yet been clarified. The purpose of this investigation was to demonstrate the usefulness of PLC in the precise staging of patients with such an occult pleural dissemination.

MATERIALS AND METHODS

Patients

A multi-institutional retrospective database analysis was performed to identify patients with lung cancer who underwent

operation between 2000 and 2007. Patients with obvious malignant pleural effusion or with Stage IV disease were excluded before the registration. A total of 3493 patients were registered from 12 institutes in which PLC had been routinely examined. After excluding the patients against eligible criteria (small cell carcinoma, 40; low-grade malignancy, 4; multiple primary lung cancer or pulmonary metastasis, 20; M1a or M1b, 62; incomplete data, 136), a total of 3231 patients were included in the study.

Methods

PLC was performed by washing the thoracic cavity with 20–500 ml of physiological saline immediately after opening the thoracic cavity during surgery; a 10–20 ml of specimen was collected for cytological examination. Actually, in most institutes, physiological saline of ≤100 ml was used for lavage fluid. Washing with 500 ml, which was used in two institutes, may increase the false-negative findings due to the over-dilution. However, according to the result of preliminary analysis that incidence of positive PLC findings per each institutes had no statistical difference ($P = 0.208$), we accepted the registration from the 500 ml institutes. We recommend the amount of lavage fluid not to exceed 100 ml. In this study, a routine radical operation for lung cancer, with mediastinal lymph node dissection conforming to the *General Rule for Clinical and Pathological Record of Lung Cancer* (6th edition) by the Japanese Lung Cancer Society [15], was performed in all patients irrespective of their PLC results. In cases where parietal pleural invasion was identified, combined resection of the pleura and chest wall, if necessary, was performed. Postoperative pathological evaluation was performed by each institute's pathologist to determine the histology, tumour size and pathological TNM. pleural invasion was also evaluated by the pathologist as a PL score ranging from PL0 to PL3 as follows: PL0, tumour within the subpleural lung parenchyma; PL1, invasion beyond the elastic layer; PL2, invasion to the pleural surface; PL3, invasion to the parietal pleura [1]. Data were collected from databases, including the result of PLC, age, gender, survival time, dead or alive (all death or censored), operative procedure, actual disease-free time, site of recurrence and information about adjuvant chemotherapy. The pathological T (pT) and pathological N (pN) scores were converted to the new 7th Edition TNM Classification [16, 17], but some stage migration of the N score could not be avoided because of the discontinuity between the Naruke map and the Rusch–Asamura map [18].

Statistical analyses

In a background analysis, age, gender, histology, pathological stage (p-Stage), pT, pN and PL scores were compared between the PLC-positive (PLC^+) group and the PLC-negative (PLC^-) group. Differences were assessed statistically using a *t*-test for the numerical variables and a χ^2 test for the categorical variables. A *P*-value of <0.05 was considered to be statistically significant. Survival analysis was performed first with the entire cohort; next, subset analyses were performed on the histology (adenocarcinoma, squamous cell carcinoma and others), p-Stage, pT, pN and PL scores. Survival curves were generated via the Kaplan–Meier method, and statistical differences between the PLC^+ group and PLC^- group were evaluated by the logrank test. A multivariate analysis using a Cox proportional hazard model (Cox analysis) was also performed to evaluate the significance of prognostic factors (PLC, age, gender, tumour size, pN and PL scores), and the hazard ratio, likelihood ratio χ^2 statistic (χ^2) and *P*-value (probability > χ^2) were estimated. All statistical analyses were performed using StatMate IV software (ATMS, Tokyo, Japan) or JMP 8.0 software (SAS Institute Japan, Tokyo, Japan).

Integration of the pleural lavage cytology-positive findings with the existing staging factors

After the evaluation of the six prognostic factors, integration of the PLC^+ findings with the existing staging factors was attempted for convenience of TNM staging. According to the results of the subset analysis and theoretical considerations, integration of the PLC^+ findings with the PL score was considered to be most reasonable. Seeking the appropriate PL score matching to the PLC^+ findings, the Cox analysis was re-estimated using a corrected PL score by replacing the score of underestimated cases with a higher score in a gradual manner (PL1, PL2 and then PL3). The

Table 1: Patient characteristics of studied groups

	PLC^+ group	PLC^- group	*P*-value
Age [mean (SD)]	66.9 (10.2)	65.6 (9.9)	0.118
Gender[a]			
Male	81 (54.7%)	1929 (62.6%)	0.054
Female	67 (45.3%)	1154 (37.4%)	
Histology[a]			
Adenocarcinoma	111 (75.0%)	2137 (69.3%)	0.015[b]
Adenosquamous cell carcinoma	6 (4.1%)	83 (2.7%)	
Squamous cell carcinoma	22 (14.9%)	752 (24.4%)	
Large cell carcinoma	5 (3.4%)	88 (2.9%)	
LCNEC	2 (1.4%)	9 (0.3%)	
Others	2 (1.4%)	14 (0.5%)	
Pathological stage[a]			
IA	24 (16.2%)	1114 (36.1%)	<0.001[b]
IB	51 (34.5%)	924 (30.0%)	
IIA	16 (10.8%)	343 (11.1%)	
IIB	11 (7.4%)	184 (6.0%)	
IIIA	43 (29.1%)	492 (16.0%)	
IIIB	3 (2.0%)	26 (0.8%)	
Pathological T score[a]			
T1a	15 (10.1%)	772 (25.0%)	<0.001[b]
T1b	13 (8.8%)	494 (16.0%)	
T2a	86 (58.1%)	1350 (43.8%)	
T2b	9 (6.1%)	147 (4.8%)	
T3	20 (13.5%)	273 (8.9%)	
T4	5 (3.4%)	47 (1.5%)	
Pathological N score[a]			
N0	91 (61.5%)	2298 (74.5%)	<0.001[b]
N1	15 (10.1%)	351 (11.4%)	
N2	41 (27.7%)	422 (13.7%)	
N3	1 (0.7%)	12 (0.4%)	
Pathological PL score[a]			
PL0	44 (29.7%)	1739 (56.4%)	<0.001[b]
PL1	45 (30.4%)	917 (29.7%)	
PL2	43 (29.1%)	246 (8.0%)	
PL3	16 (10.8%)	181 (5.9%)	

PLC: pleural lavage cytology; PLC^+: PLC-positive group; PLC^-: PLC-negative group; LCNEC: large cell neuroendocrine carcinoma.
[a]Expressed by the number of the cases with its ratio.
[b]Statistical difference was confirmed with $P < 0.05$.

reliability of each Cox proportional hazard model was evaluated by the χ^2 and *P*-value with regard to the whole model and to the PL score. Since the *P*-values were too small to compare with each other, χ^2 was used in this instance. The model with the largest χ^2 has the smallest *P*-value and, therefore, is the most reliable model.

RESULTS

The incidence of PLC$^+$ findings was 4.58% (148/3231). In a background analysis, histology, pathological stage (p-Stage), pT, pN and PL scores had significant differences between the groups (Table 1). It was suspected that the PLC$^+$ group consisted of patients whose cancer had advanced to a particular stage. Regarding to the higher incidence of N2 disease in the PLC$^+$ group, cancer may migrate the lymphatic channels of the pleura and may cause the lymph node metastasis. However, the recurrence rate associated with the mediastinal-supraclavicular lymph node enlargement had no statistical difference ($P = 0.450$) between the PLC$^+$ group and the PLC$^-$ group, which was estimated to be 8.8 and 6.6%, respectively. The survival curve of the PLC$^+$ group was significantly worse than that of the PLC$^-$ group in terms of both the overall survival (OS) and disease-free survival (DFS) (Fig. 1). Differences in the subset analysis are shown in Table 2, and DFS curves for each p-Stage and each PL score are shown in Figs 2 and 3, respectively. In Stages IA and IB, the survival curves of the PLC$^+$ group were significantly worse than those of the PLC$^-$ group. As for the PL score, the survival curves of the PLC$^+$ group were also worse in the PL0 and PL1 groups. However, differences were not observed in the PL2 and PL3 groups. These findings suggested that the PLC$^+$ patients should not be included in these earlier stages.

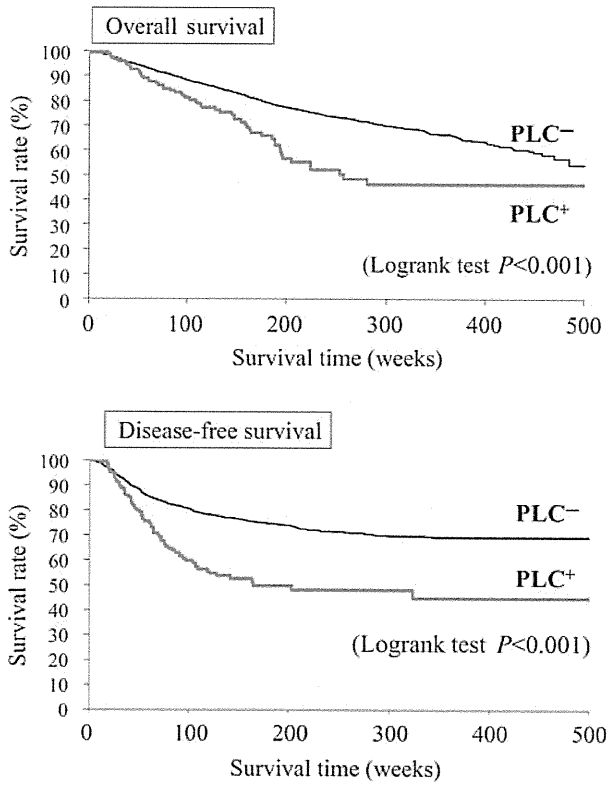

Figure 1: Comparison of survival curves by PLC status. Overall survival curves are shown in the top panel and DFS curves in the bottom panel. Statistical differences (*P*-values) were calculated by the logrank test. PLC: pleural lavage cytology; PLC$^+$: PLC-positive group; PLC$^-$: PLC-negative group.

Table 2: Differences in survival between the PLC$^+$ group and the PLC$^-$ group

	Overall survival	Disease-free survival
Histology		
Adenocarcinoma	$P < 0.001$*	$P < 0.001$*
Squamous cell carcinoma	$P = 0.496$	$P = 0.188$
Others	$P = 0.877$	$P = 0.837$
Pathological stage		
IA	$P = 0.045$*	$P < 0.001$*
IB	$P = 0.010$*	$P < 0.001$*
IIA	$P = 0.821$	$P = 0.270$
IIB	$P = 0.004$*	$P = 0.003$*
IIIA	$P = 0.984$	$P = 0.993$
IIIB	$P = 0.984$	$P = 0.149$
Pathological T score		
T1a	$P = 0.928$	$P = 0.025$*
T1b	$P = 0.094$	$P = 0.009$*
T2a	$P = 0.023$*	$P < 0.001$*
T2b	$P = 0.668$	$P = 0.923$
T3	$P = 0.273$	$P = 0.151$
T4	$P = 0.204$	$P = 0.783$
Pathological N score		
N0	$P < 0.001$*	$P < 0.001$*
N1	$P = 0.281$	$P = 0.023$*
N2+3	$P = 0.472$	$P = 0.351$
Pathological PL score		
PL0	$P = 0.129$	$P = 0.013$*
PL1	$P = 0.026$*	$P < 0.001$*
PL2	$P = 0.184$	$P = 0.079$
PL3	$P = 0.948$	$P = 0.875$

Expressed by *P*-values of the logrank test.
PLC: pleural lavage cytology; PLC$^+$: PLC-positive group; PLC$^-$: PLC-negative group.
*Statistical difference was confirmed with $P < 0.05$.

In an analysis of recurrent cases, the incidence of a malignant pleural effusion or obvious pleural dissemination (pleuritis carcinomatosa) was 17.6% (26/148) in the PLC$^+$ group, compared with 2.8% (86/3083) in the PLC$^-$ group, a significant difference ($P < 0.001$). However, no difference was apparent with regard to sites of distant metastasis. For this reason, it was concluded that PLC$^+$ findings was a preliminary stage of a malignant pleural effusion.

Among the six variables analysed by Cox analysis, all were statistically significant in terms of OS and DFS (Table 3). PLC$^+$ findings were confirmed as a significantly poor prognostic factor in both OS ($P = 0.016$) and DFS ($P = 0.026$). However, it would be more convenient if the PLC$^+$ findings were integrated with one of the existing TNM staging factors. A total of 89 cases (60.1%) with PLC$^+$ findings had been diagnosed as either PL1 or PL0, which the subset analysis showed to be underestimations of the disease stage. To find the accurate PL score for positive PLC findings, the Cox analysis was re-estimated using the PL score upgraded stepwise. The χ^2 regarding to the whole model reached its maximum value by a correction to PL3 in both OS (uncorrected, PL2, PL3; 654.67, 658.99, 659.04) and DFS

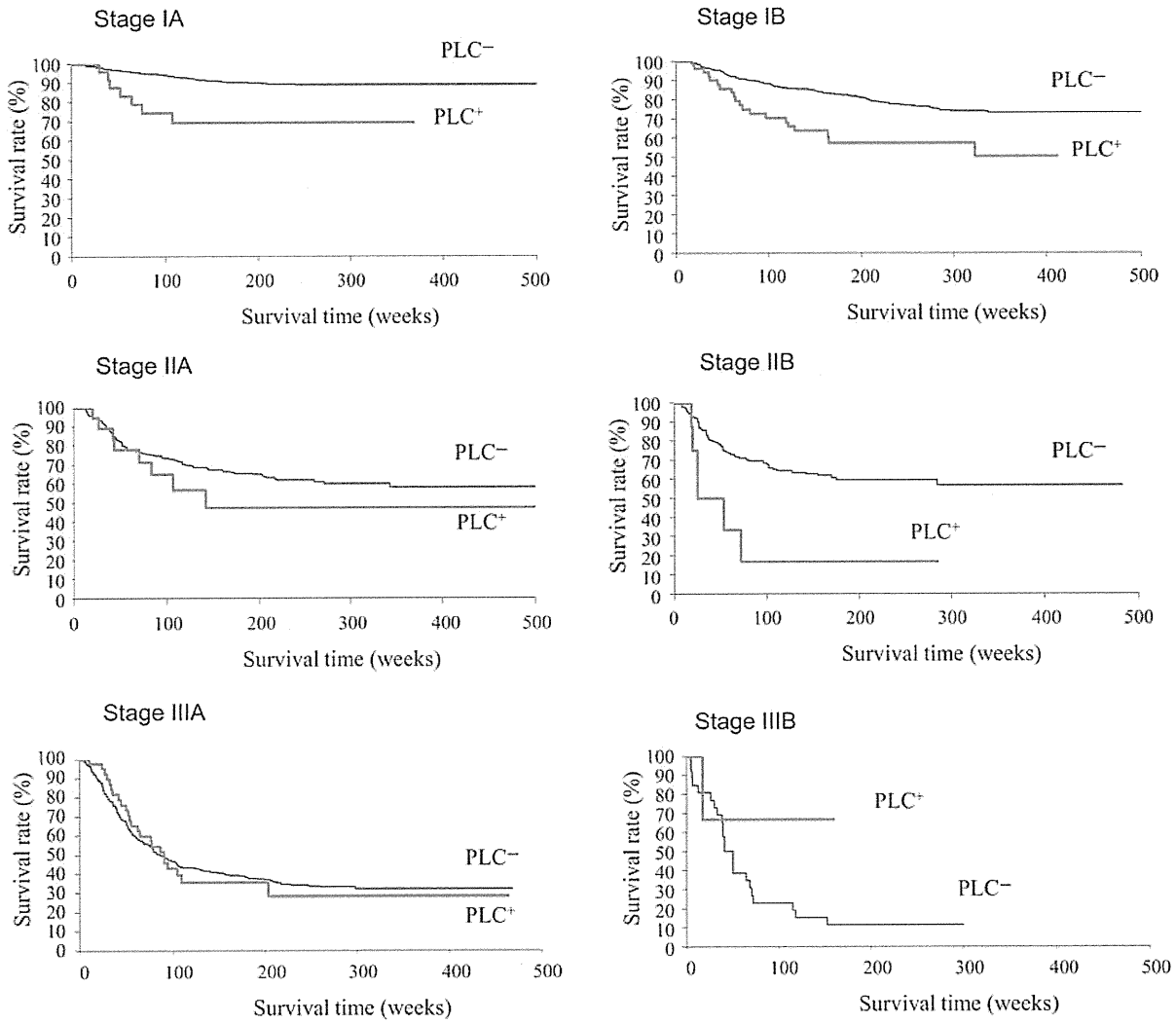

Figure 2: Comparison of DFS curves by pathological stage. Statistical differences (*P*-values) were calculated by the logrank test. PLC: pleural lavage cytology; PLC⁺: PLC-positive group; PLC⁻: PLC-negative group.

(uncorrected, PL2, PL3; 600.56, 609.28, 609.84). Conversion of the PLC⁺ findings to PL3 (=T3) was, therefore, considered to be most appropriate. DFS curves that were re-estimated using the corrected PL score are shown in Fig. 4 to demonstrate the efficacy of correction.

DISCUSSION

The previously reported incidence of PLC⁺ findings ranges from 2.7 to 41.7% [2–14]. However, restricting to the papers of large series, the incidence of PLC⁺ findings was found to be within the range of 3–6%. PLC⁺ findings were reasonably estimated to be 4.58% in our study. Although the survival differences between the PLC⁺ and PLC⁻ groups are obvious, these differences may not have been due only to the sequelae of PLC⁺ findings, because many of the other patient characteristics were also significantly different. For this reason, a Cox analysis was performed. All of the six variables analysed were statistically significant and PLC⁺ findings were confirmed as a significantly poor prognostic factor. As for the results of the Cox analysis, many investigators [2, 4, 7, 8, 10, 12, 14] have reported that PLC⁺ findings are an independent prognostic factor in lung cancer. However, their analysed explanatory variables are inconsistent. Above all, pN, which is widely believed to be the most important prognostic factor, is not included in many studies [2, 4, 7, 8, 10]. In some study, it is converted to a much rougher score, such as 'N0 vs. N1–3' [12]. In our study, the explanatory variables were simplified into two categories, one concerning the life expectancy (age and gender) and the other concerning the tumour growth (tumour size, pN and PL score); p-Stage and pT were not included because these factors may depend on other factors. We used the raw values of pN and PL score. If either of these scores was excluded from the explanatory variables, PLC⁺ findings acquire a much smaller *P*-value ($P = 0.001$/OS without pN, $P < 0.001$/OS without PL score, $P < 0.001$/DFS without pN, $P < 0.001$/DFS without PL score) and will be regarded as a much more important prognostic factor. However, this is nothing more than a statistical artefact. The impact of PLC⁺ findings should not be overstated. We were simply analysing a particular stage of cancer progression.

The extent of pleural invasion is expressed by a pleural invasion score ranging from PL0 to PL3 and is considered to be useful in

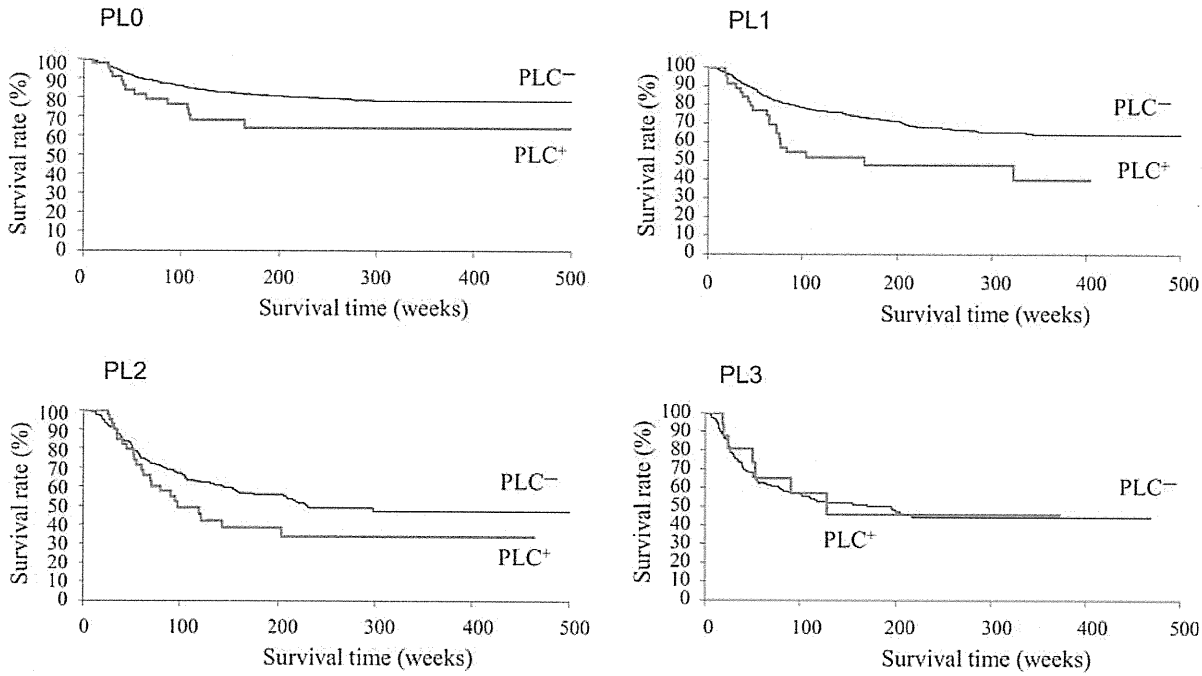

Figure 3: Comparison of DFS curves by the PL score. Statistical differences (*P*-values) were calculated by the logrank test. PLC: pleural lavage cytology; PLC⁺: PLC-positive group; PLC⁻: PLC-negative group.

Table 3: Results of the Cox analysis for overall survival and DFS

Factors	Hazard ratio	Likelihood ratio, χ^2	*P*-value
PLC⁺		5.848 (4.930)	0.016 (0.026)
PLC⁺/PLC⁻	1.436 (1.361)		
Age	1.037 (1.008)	83.419 (4.653)	<0.001 (0.031)
Gender		79.221 (9.458)	<0.001 (0.002)
Male/Female	2.107 (1.265)		
Tumour size	1.014 (1.012)	47.090 (34.032)	<0.001 (<0.001)
N score		227.301 (326.769)	<0.001 (<0.001)
N1/N0	2.036 (2.333)		
N2/N0	3.591 (4.546)		
N3/N0	7.253 (6.579)		
PL score		46.667 (46.140)	<0.001 (<0.001)
PL1/PL0	1.116 (1.343)		
PL2/PL0	1.695 (1.810)		
PL3/PL0	2.079 (2.001)		

Results for DFS data are shown in parentheses.
Cox analysis: multivariate analysis using the Cox proportional hazard model; PLC: pleural lavage cytology; PLC⁺: PLC-positive group; PLC⁻: PLC-negative group.

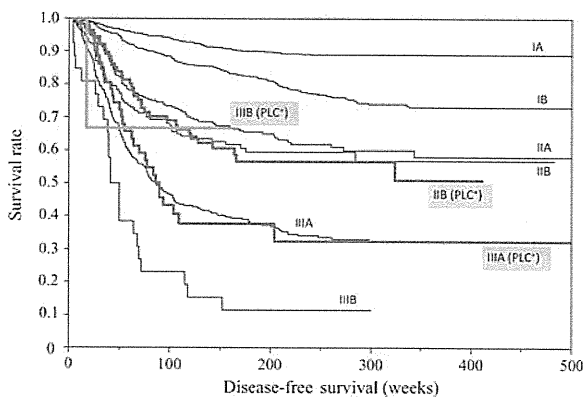

Figure 4: DFS curves after re-staging of the PLC⁺ patients, shown in comparison with that of the PLC⁻ patients. PLC: pleural lavage cytology; PLC⁺: PLC-positive; PLC⁻: PLC-negative; IIB (PLC⁺): Stage IIB in the PLC-positive group; IIIA (PLC⁺): Stage IIIA in the PLC-positive group; IIIB (PLC⁺): Stage IIIB in the PLC-positive group.

predicting prognosis [1]. PL3 is classified as T3 in the TNM classification; recently, PL1 and PL2 were classified as T2a or T2b (depending on tumour size) in the 7th Edition TNM classification [16, 17]. Moreover, in the 7th Edition TNM classification system, the classification of a malignant pleural effusion (pleuritis carcinomatosa) increased from T4 to M1a [16, 17] because of its vicious prognosis. Before the appearance of a pleural effusion, occult (microscopic) dissemination must occur. Although this stage is not currently evaluated, it is detectable by the cytological examination of the pleural cavity, such as via PLC. Theoretically, patients with PLC⁺ findings must be given a score of PL2 or higher because the cancer cells were exfoliated from the lung surface. However, 60.1% of the cases had been diagnosed as either PL1 or PL0 in our study. There is a discrepancy between the theory and clinical data. To evaluate the reliability of the staging, subset analysis was performed. In Stages IA and IB, the survival curves of the PLC⁺ group were significantly worse than those of the PLC⁻ group. As for the PL score, the survival curves of the PLC⁺ group were also worse in the PL0 and PL1 groups. These findings suggested that the PLC⁺ patients should not be included in these stages; instead, they should be classified in more advanced stages. As for the cause of discrepancy in the PL score, two possible explanations are conceivable: (i) cancer cells in the pleural cavity came from another origin, for example, exudation from the

lymphatic channels or nodes; (ii) diagnosis of PL0 or PL1 was made using inappropriate section of histopathological specimen, for example, in the case with deep pleural indentation. The former is a most likely explanation, but it cannot be a single credible cause, because the ratio of N1–2 patients per PL0–1 patients in the PLC^+ group was only 31% in our data. We cannot get farther information because of the limitation of retrospective study. Although cancer cells in the pleural cavity do not always originate from the lung surface, microscopic dissemination should be recognized as a preliminary stage of the malignant pleural effusion. This is the reason why we proposed the re-staging by PLC^+ findings.

Although the PLC^+ findings were confirmed as a significantly poor prognostic factor in the Cox analysis, it would be more convenient if the PLC^+ findings were integrated with one of the existing TNM staging factors. Integration of PLC^+ findings into the PL score may positively contribute to the precise diagnosis of cancer advancement and, therefore, will be useful in evaluating its prognosis. Scoring PLC^+ findings as PL3 (=T3) should be a reasonable method to express the stage between PL2 (=T2a–b) and T4 (=M1a).

Standard operation for lung cancer should not be given up because of the positive findings of PLC. The DFS of the PLC^+ patients, whose stages were re-staged to be either IIB (T3N0) or IIIA (T3N1 and T3N2), were almost equal with that of the ordinary (PLC^- group) Stage IIB or IIIA patients. Their survival is much better than that of the patients with malignant pleural effusion. Although we could not prove the efficacy of adjuvant therapy, due to the retrospective clinical data analysis, adjuvant chemotherapy will be indispensable. Intra-operative intra-pleural administration of hypotonic cysplatin [19] is a procedure of great interest. But farther investigations will be necessary to establish its efficacy.

CONCLUSION

Examining PLC in clinical practice is useful for detecting occult pleural dissemination before the appearance of a malignant pleural effusion. Evidence of PLC^+ findings should be treated as supplemental information to the precise diagnosis of PL score. Scoring PLC^+ findings as PL3 (=T3) was appropriate. However, standard operation should not be given up because of the positive PLC findings. The corrected survival curves of the PLC^+ group were almost equal with that of the ordinary stage IIB or IIIA patients.

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Conflict of interest: none declared.

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