Statistical Analysis

The sample size was calculated as follows. Given that the 5-year survival rate would be 70% in the surgery-only group, with an HR of 0.70, $\alpha = .05$ (two-sided), and a statistical power of 80%, we estimated that 1,000 patients would be required. OS and RFS were estimated on the basis of all randomly assigned patients. The results in eligible patients were analyzed according to disease stage. OS was defined as the interval from the date of random assignment to the date of death from any cause. RFS was defined as the interval from the date of random assignment to the date of confirming recurrence or death from any cause, whichever came first. Data for up to 5 years from the date of random assignment were analyzed. Data obtained after 5 years were not included in this analysis. The survival rate was estimated by using the Kaplan-Meier method. The Cox proportional hazards model was used to calculate HRs. All statistical analyses were done with SAS, version 9.1 (SAS Institute, Cary, NC).

RESULTS

Patients

From October 2001 through December 2004, a total of 1,059 patients were enrolled at 109 centers throughout Japan; 529 were assigned to the S-1 group and 530 to the surgery-only group (intention-to-treat population; Fig 1). In both groups combined, 474 patients (44.8%) had stage II disease, 409 (38.6%) had stage IIIA disease, and 175 (16.5%) had stage IIIB disease. The numbers of patients with each stage of disease were similar in the two treatment groups. The groups were also well balanced with respect to the type of gastrectomy performed, the combined resection of other organs, and other factors. Details of the patient demographics and baseline characteristics have been reported previously.[12]

Fourteen patients in the S-1 group and 11 in the surgery-only group were ineligible, as shown in Figure 1. In the S-1 group, 12 patients did not receive S-1. In the surgery-only group, four patients received adjuvant treatment at their strong request, violating the protocol.

Safety

Details of the safety analysis have been reported previously.[12] In brief, except for anorexia (incidence, 6%), grade 3 or 4 adverse events occurred in less than 5% of the patients in the S-1 group.

OS and RFS in All Randomly Assigned Patients

Among 1,059 patients, 145 and 199 died, 32 and 42 patients are alive with recurrence, and 352 and 289 patients are alive without recurrence in the S-1 and the surgery-only groups, respectively. Data on 131 patients lost to follow-up within 5 years from the date of random assignment were censored.

OS and RFS were analyzed in all 1,059 randomly assigned patients. The 5-year OS rate was 71.7% (95% CI, 67.8% to 75.7%) in the S-1 group and 61.1% (95% CI, 56.8% to 65.3%) in the surgery-only group. The HR for death in the S-1 group compared with the surgery-only group was 0.669 (95% CI, 0.540 to 0.828), indicating that S-1 reduced the risk of death by 33.1% (Fig 2A). The 5-year RFS rate was 65.4% (95% CI, 61.2% to 69.5%) in the S-1 group and 53.1% (95% CI, 48.7% to 57.4%) in the surgery-only group. The HR for relapse in the S-1 group compared with that in the surgery-only group was 0.653 (95% CI, 0.537 to 0.793). Treatment with S-1 thus reduced the risk of relapse by 34.7% (Fig 2B).

Subgroup Analysis

OS and RFS in eligible patients were analyzed according to sex, age, disease stage (Japanese Classification, 13th edition), and histologic type. There was no interaction between treatment and any of these factors (Fig 3). Kaplan-Meier estimates of OS and RFS are shown according to disease stage, which was used as a stratification factor when patients were randomly assigned (Figs 4, 5, and 6).

The 5-year OS rates of the patients with stage II disease were 84.2% (95% CI, 79.5% to 89.0%) in the S-1group and 71.3% (95% CI, 65.3% to 77.2%) in the surgery-only group, with an HR of 0.509 (95% CI, 0.338 to 0.765; Fig 4A). Their 5-year RFS rates were 79.2% (95% CI, 73.8% to 84.6%) in the S-1 group and 64.4% (95% CI, 58.1% to 70.7%) in the surgery-only group, with an HR of 0.521 (95% CI, 0.362 to 0.750; Fig 4B). The 5-year OS rates of stage IIIA patients were 67.1% (95% CI, 60.4% to 73.8%) in the S-1 group and 57.3% (95% CI, 50.3% to 64.2%) in the surgery-alone group, with an HR of 0.708 (95% CI, 0.510 to 0.983; Fig 5A). Their 5-year RFS rates were 61.4% (95% CI, 54.5% to 68.4%) in the S-1 group and 50.0% (95% CI, 42.9% to 57.0%) in the surgery-alone group, with an HR of 0.696 (95% CI,

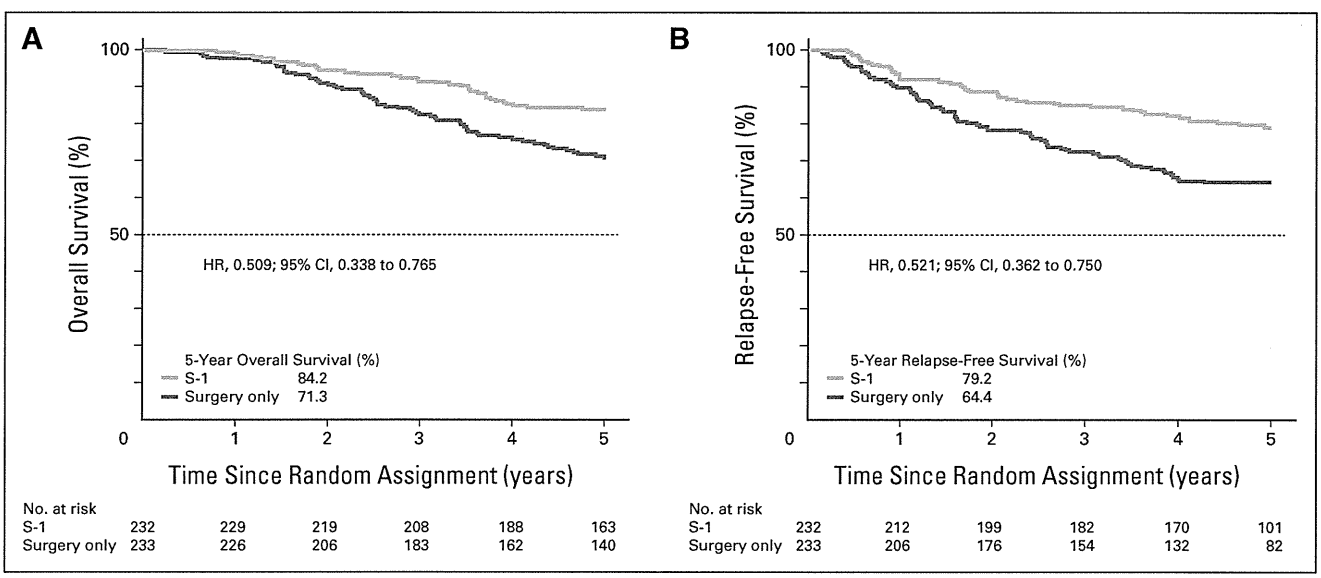

Fig 4. Kaplan-Meier estimates of (A) overall survival and (B) relapse-free survival for eligible patients with stage II gastric cancer. HR, hazard ratio.

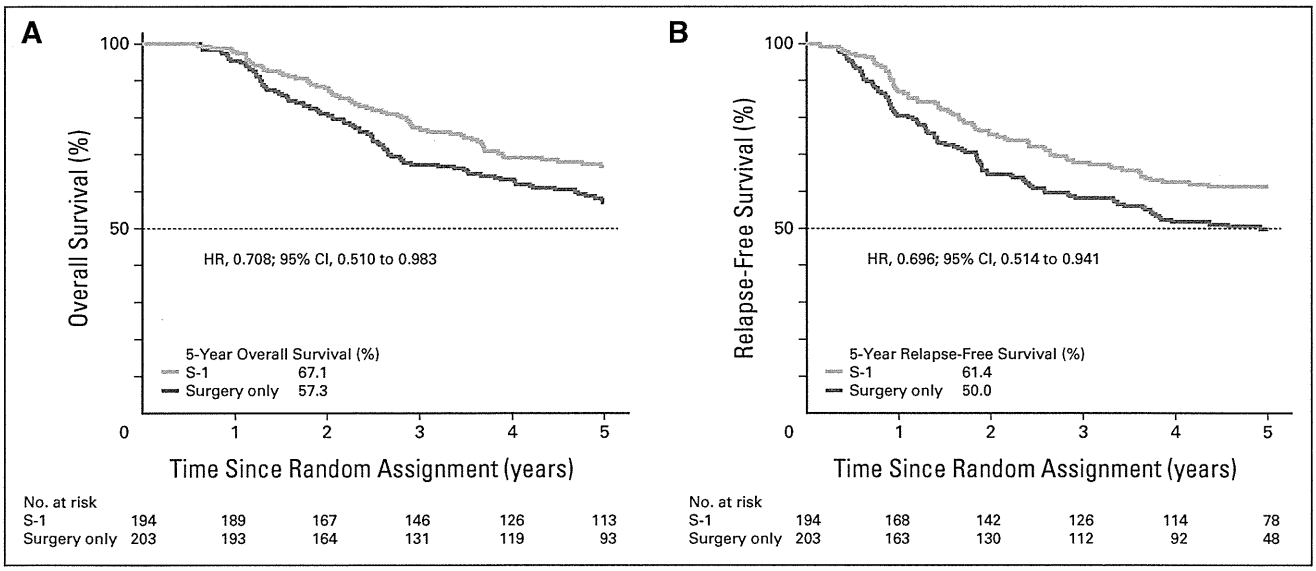

Fig 5. Kaplan-Meier estimates of (A) overall survival and (B) relapse-free survival for eligible patients with stage IIIA gastric cancer. HR, hazard ratio.

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.

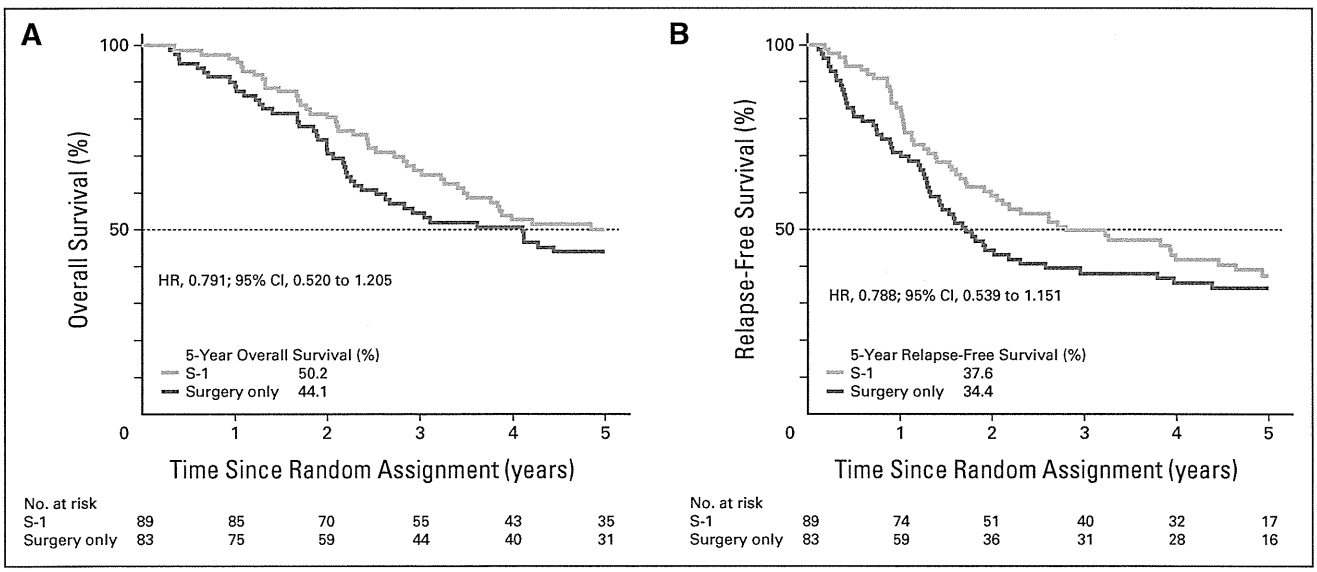

Fig 6. Kaplan-Meier estimates of (A) overall survival and (B) relapse-free survival for eligible patients with stage IIIB gastric cancer. HR, hazard ratio.

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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Gastric Cancer Eastern Experience

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KEYWORDS

• Gastric cancer surgery • D2 dissection • Stage migration
• Quality of lymphadenectomy • Quality of postoperative care
• Splenectomy

GUIDELINES FOR THE STANDARD TREATMENT OF GASTRIC CANCER

Several guidelines are used for cancer therapy throughout the world. In the Japan Gastric Cancer Association (JGCA) guideline, standard surgery for T2 to T4 curable gastric cancer is defined as more than two-thirds gastrectomy with D2 dissection.[1] In the 2010 European Society of Medical Oncology's guideline, the standard surgery for curable gastric cancer is the D2 gastrectomy.[2] Of note, this is the first time this society has clearly advocated for the D2 approach. The National Comprehensive Cancer Network (NCCN) guidelines, commonly followed in the United States, recommend that gastric resections include regional lymphadenectomy to include the perigastric lymph nodes (D1) and those along the named vessels of the celiac axis (D2), with a goal of examining at least 15 or more lymph nodes.[3]

STAGE-SPECIFIC RESULTS OF RESECTED GASTRIC CANCER IN THE WEST AND EAST

The JGCA-maintained registry analyzed a total of 11,261 patients who underwent gastric resection in 2001.[4] The 5-year overall survival (OS) by UICC TNM stage (sixth version) was as follows: stage IA, 91.8%; stage IB, 84.6%; stage II, 70.5%; stage IIIA, 46.6%; stage IIIB, 29.9%; stage IV, 16.6%. Although the standard treatment at that time was surgery alone[5], an unknown proportion of those undergoing surgery may also have received adjuvant treatment either through enrollment into clinical trials or by doctor's or patient's choice.

Another available source of information regarding gastric cancer survival is obtained through single-institution reporting. Five-year OS after a total gastrectomy of 881 patients undergoing a total gastrectomy between 1995 and 2001 at Asan Medical Center, Korea, was 94.6%, 90.8%, 76.7%, 55.7%, 41.3%, and 15.4% for stage IA, IB, II, IIIA, IIIB, and IV, respectively.[6] From another Korean institution, National Seoul University Hospital, the results of 10,783 consecutive patients who were surgically treated

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between 1970 and 1996 were reported. Five-year OS was 92.9%, 84.2%, 69.3%, 45.8%, 29.6%, and 9.2% for stage IA, IB, II, IIIA, IIIB, and IV, respectively.[7] Differences in these results seem attributable mainly to the period of inclusion and improvement over time. Selection bias hampers straight comparison with nationwide registry.

The nationwide results of the United States by the National Cancer Data Base (NCDB) were reported for the cohort treated between 1985 and 1996.[8] Stage-specific OS was 78%, 58%, 34%, 20%, 8%, and 7% for stage IA, IB, II, IIIA, IIIB, and IV, respectively (**Table 1**). More recent data, after the results of Intergroup study 0116 (INT 0116), have yet to be published in medical journals. According to the report by Enestvedt and colleagues,[9] 36.8% of patients surgically staged from IB to III underwent adjuvant chemoradiotherapy after gastric resection between 2001 and 2006 in the state of Oregon. Stage-specific 5-year OS was approximately 13%, 13%, and 5% for stage IB, II, and III, respectively. These results are unacceptably poor, explained by the extremely low percentage of proper adjuvant treatment, correct staging, or adequate surgery. With this kind of data base it is not easy to obtain the precise details of patients' background, and comparison is not easy.

OVERALL SURVIVAL IN VARIOUS CLINICAL TRIALS IN THE WEST AND EAST

To know exactly the stage-specific OS by surgery alone, the most reliable way is to analyze the results of the surgery-alone arm of clinical trials that have evaluated some kind of new treatment in comparison with a surgery-alone arm as control. Since 2007, when the results of INT-0116,[10] the MAGIC trial,[11] and ACTS-GC[12] became available, it has become difficult to carry out a randomized controlled trial (RCT) having surgery alone as control.

In Japan the results of the surgery-alone arm of the ACTS-GC study, in which 1059 patients were enrolled, are available. In this trial, only stage II and IIIA/B by the Japanese classification were included. These patients can be restaged by UICC TNM classification. Some patients in stage III in the Japanese classification were classified as stage IV by TNM classification. Five-year OS was 70.8%, 56.2%, 40.1%, and 42.7% for UICC stage II, IIIA, IIIB, and IV, respectively in the surgery-only group.[13] In the Dutch Gastric Cancer Study, the 5-year OS was 81%, 61%, 42%, 28%, 13%, and 28%, for stage IA, IB, II, IIIA, IIIB, and IV, respectively.[14] Although the Italian Gastric Cancer Study was a phase 2 study, they reported stage-specific survival due to a larger number of patients included.[15] As shown in **Table 2**, their results are somewhere between those of the ACTS-GC and the Dutch study.

Table 1
Stage-specific 5-year overall survival rate (%) of cancer registries and a large case series

	JGCA Registry	SNUH	NCDB
Period	2001	1970–1996	1985–1996
Stage IA	91.8	92.9	78
Stage IB	84.6	84.2	58
Stage II	70.5	69.3	34
Stage IIIA	46.6	45.8	20
Stage IIIB	29.9	29.6	8
Stage IV	16.6	9.2	7
Total patients	11261	10783	49756

Abbreviations: JGCA, Japan Gastric Cancer Association; NCDB, National Cancer Data Base; SNUH, Seoul National University Hospital.

Table 2
Stage-specific 5-year overall survival rate (%) of D2 surgery in clinical trials

	ACTS-GC[13]	Dutch D1 vs D2[14]	Italian P2[15]
Stage IA		81 (69)	95.0 (53)
Stage IB		61 (64)	87.5 (22)
Stage II	70.2 (278)	42 (66)	57.5 (31)
Stage IIIA	56.2 (153)	28 (72)	42.5 (37)
Stage IIIB	40.1 (53)	13 (39)	22.5 (25)
Stage IV	42.7 (35)	28 (18)	2.5 (23)
Total patients	519	328	191

Numbers in parentheses show number of patients for each stage.
Abbreviation: ACTS-GC, Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer.

In other clinical trials,[16–19] stage-specific OS cannot be obtained in publications but they would not be reliable, if available, because of the small numbers in each stage in these trials as compared with the ACTS-GC. Careful comparison of the patients' background may suggest some difference in these results. **Table 3** shows the background of the patients enrolled in the surgery-alone arm of these studies. Compared with the results of Western trials, much better OS are shown in Japanese trials (see **Table 3**).

STAGE MIGRATION: FACT AND SOURCE OF MIGRATION

Stage migration is a hampering factor when trying to compare the stage-specific results of different countries where the accuracy of staging is different. Wider lymph node dissection and more accurate lymph retrieval from the specimen result in more accurate staging, which in turn results in better stage-specific survival. Bunt and colleagues[20] evaluated the effect of stage migration in the Dutch study where D1 and D2 dissection were compared. If the patients who underwent D2 dissection were restaged abandoning the information about N2 level, 72 of 214 (34%) would have a different stage due to stage migration. Using the reported Japanese stage-specific survival results, calculated stage-specific survival by D2 staging is better in each stage than that of calculated stage-specific survival if N2 information is not used for staging. Especially in stage IIIA and IIIB, as much as 15% difference could be expected between these two staging systems. In the Japan Clinical Oncology Group (JCOG) study 9501 where D2 and D2+ para-aortic node dissection were compared, similar stage migration was observed. However, the incidence of para-aortic node metastasis (8.8%) is much smaller than that of N2 nodes, therefore only 8.5% of the entire patient cohort who underwent D3 dissection could have been restaged by abandoning the N3 information.[21]

In the Dutch study it was found that not only the extent of nodal dissection but also the way of retrieving nodes and the effort of pathologists resulted in stage migration.[22] Similarly, how the resected stomach is examined may be a source of stage migration. If the deepest part of the region is not histologically examined, earlier T stage would be attributed to these lesions.

SPLENECTOMY

In both the Dutch and the Medical Research Council (MRC) study comparing D1 with D2 surgery, splenectomy was found to be more relevant than D2 itself, due to higher

Table 3
Comparison of patients' characteristics, background, treatment, and 5-year overall survival in the surgery-alone arm of clinical trials

	JCOG 9206-2[16]	JCOG 9501[17]	INT-0116[10]	MAGIC[11]	EORCT 40954[18]	FNCLCC/FFCD[19]
No. of patients	133	523	275	253 (204)	72 (68)	110 (98)
Tumor location (%)						
L/M/U/W	39/44/37/12	217/206/100/0	154/69/50/0	NA	15/18/39	NA
Histological type (%)						
Dif/undif	43/88	204/316	77/128/70	NA	39/33	NA
pT stage (1/2/3/4)	2/39/88/4	23/257/230/13	22/63/168/22	16/55/106/16	4/30/24/7	27///58[a]
% pT3/4	69%	46%	65%	63%	48%	68%
pN (±)	101/32	348/175	231/44	114/42	52/13	68/17
% Node positive	76%	67%	84%	73%	80%	80%
Median size	5.5	5.5	NA	5.0	NA	NA
Surgery <D2/≧D2 (%)	0/132	0/523	254/20	70/96	5/63	NA
R0 resection	100%	100%	100%?	66%	67%	74%
5-Year OS	61%	70%	~25%	23%	~50%	24%

Abbreviations: Dif, differentiated; L/M/U/W, Distal part/Middle part/Proximal part/Whole stomach; NA, not available; OS, overall survival; undif, undifferentiated.
[a] T1 + 2///T3 + 4: numbers of T1 and T2 versus T3 and T4.

postoperative mortality.[23,24] In these trials, the protocol required the surgeons to carry out a splenopancreatectomy in case of a total gastrectomy in the D2 arm. Therefore, the majority of those who underwent total gastrectomy received splenectomy and distal pancreatectomy. Because of misunderstanding of the Japanese classification and definition of D category, even some patients who underwent a distal gastrectomy received splenectomy in these trials, which resulted in high mortality due to remnant stomach necrosis.[23]

Moreover, the worse prognosis of the D2 group was attributed to splenectomy in MRC trials comparing two groups of patients who underwent splenectomy or not.[25] However, it is known that prognosis of tumors located in the upper part of the stomach is worse than that of distally located tumors. The larger the tumor, the more frequently they require a total gastrectomy. These factors, biology of proximal tumor and size of tumors, seem to strongly affect the survival results. To avoid such bias, only an RCT comparing a total gastrectomy with and without splenectomy can provide a proper conclusion to this question. The JCOG performed an RCT to evaluate the noninferiority of spleen-preserving total gastrectomy to a pancreas-preserving total gastrectomy with splenectomy for patients who had T2 or deeper tumors in the proximal part of the stomach, requiring a total gastrectomy.[26] Sano and colleagues[27] reported more blood loss and higher morbidity after splenectomy, but no difference in mortality in experienced surgeons' hands. Long-term results are awaited.

IMPACT OF D2 DISSECTION ON THE RESULTS OF ADJUVANT TREATMENT

In the INT-0116 study, subgroup analysis by extent of lymphadenectomy revealed that the effect of adjuvant chemoradiation depends on the type of lymphadenectomy. Due to the limited number of those undergoing D2 dissection in this study, interaction between treatment effect and type of lymphadenectomy was not statistically significant, but those with D2 dissection did not show any benefit of adjuvant chemoradiation. These results were later transformed into the correlation between Maruyama Index (a computer program–based probability calculation of nodal residual disease) and the survival results of the patients in this study.[28] Dikken and colleagues[29] reported the influence of the extent of lymphadenectomy on the pattern of recurrence and OS in comparison with chemoradiotherapy. The investigators suggested that effect of chemoradiotherapy depends on type of lymphadenectomy, and that postoperative adjuvant chemoradiotherapy might compensate nonradical surgery for better local control.

Historically only two pivotal studies were able to show the benefit of adjuvant chemotherapy, the ACTS-GC study[12] and the CLASSIC study.[30] In these studies, all patients underwent D2 dissection as local control. The effect of radiotherapy added to adjuvant chemotherapy is being tested in two clinical trials.[31] The CRITICS trial is a European study launched in the Netherlands, wherein the effect of postoperative chemoradiotherapy (capecitabine + cisplatin with 45 Gy radiation) is compared with postoperative chemotherapy alone in the course of European standard perioperative treatment (preoperative chemotherapy comprising 3 courses of epirubicin + cisplatin + capecitabine and D1+ surgery followed by postoperative chemotherapy [same as the preoperative one]). This study is still open for accrual.[31] Another study is the ARTIST trial, a Korean single-institutional study, which compares postoperative adjuvant therapy by capecitabine + cisplatin with or without simultaneous radiotherapy. All patients should undergo D2 dissection. Four hundred and fifty-eight patients were enrolled between October 2004 and April 2008, and the short-term results, mainly concerning the safety profile, were reported in ASCO-GI 2009.[32] The final results are yet to be reported.

SUMMARY

In the East, D2 dissection shows much better results than less extended surgery followed by adjuvant treatment. Adjuvant chemotherapy without radiotherapy show significantly better survival results than surgery alone only when D2 dissection is applied. Without good local control, including regional lymph node metastasis, cure rate cannot be high.

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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

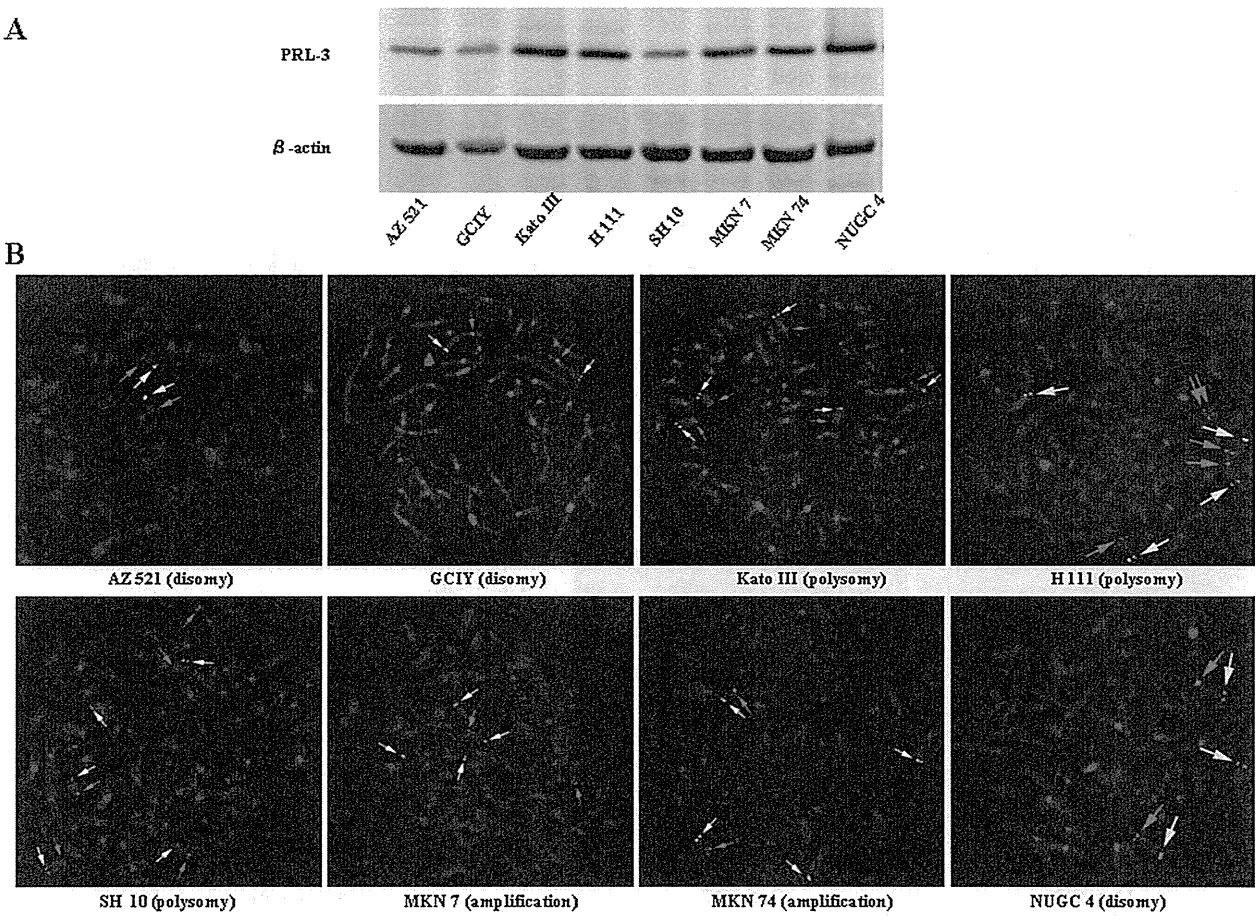

Figure 1 Expression and genetic status of PRL-3 in 8GC cell lines. (A) Expression level of PRL-3 by western blotting. (B) FISH analysis on metaphase of *PRL-3* gene (green). The chromosome 8 centromeric probe (orange) was used to estimate the copy number.

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

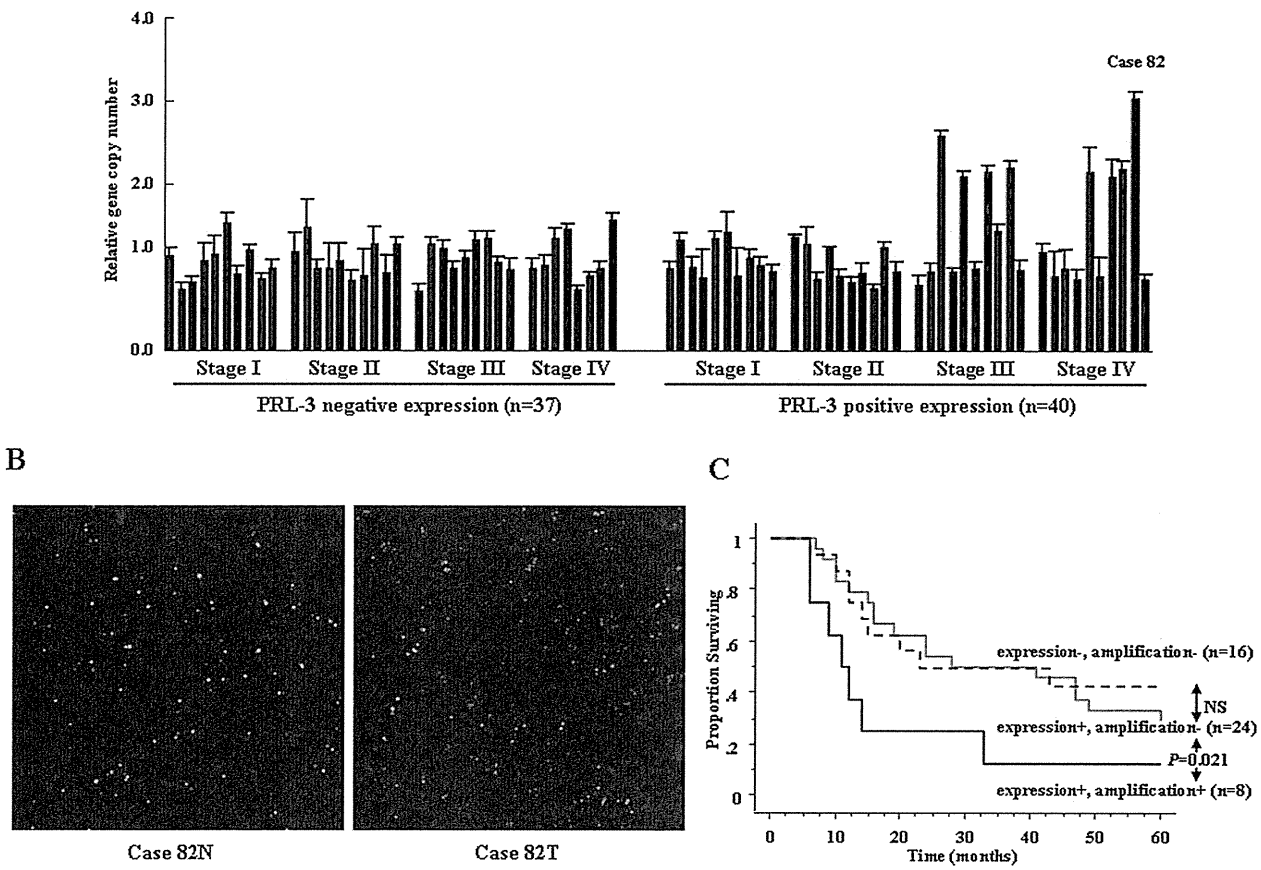

Figure 2 Frequency and prognosis of *PRL-3* genomic amplification in 77 human GC. (A) Frequency of *PRL-3* genomic amplification using Q-PCR in 77 human GC. Q-PCR was performed for both the 40 tumors with positive PRL-3 expression and 37 tumors with negative expression. To normalize *PRL-3* gene copy 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,

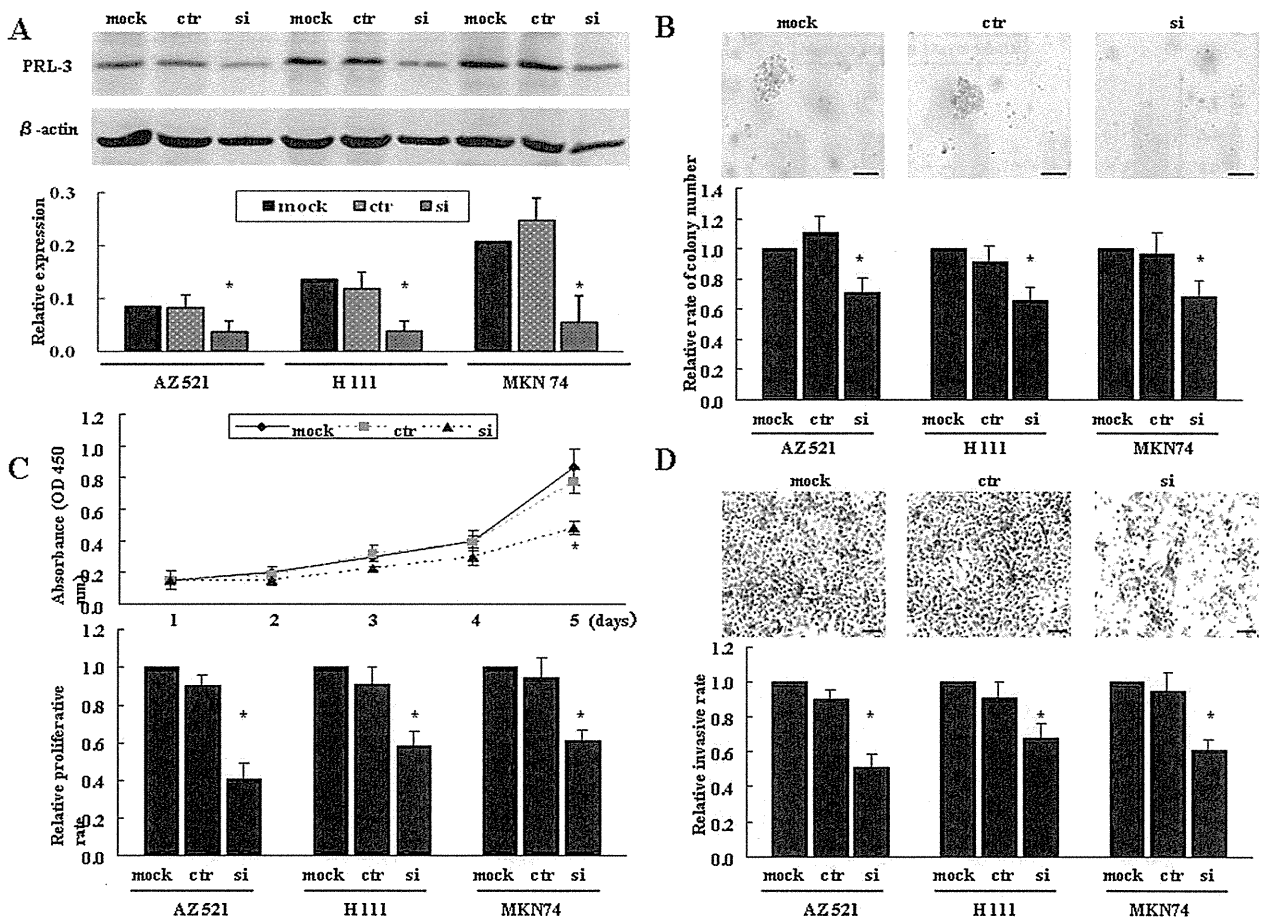

Figure 3 Inhibition of metastatic properties after transfection with siRNA in cells with expression. (A) Western blotting 72 hours after transfection. The western blotting was also quantified. mock, treatment with Accell siRNA Delivery Media alone; ctr, treatment with siRNA-ctr; si, treatment with siRNA-PRL-3. (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

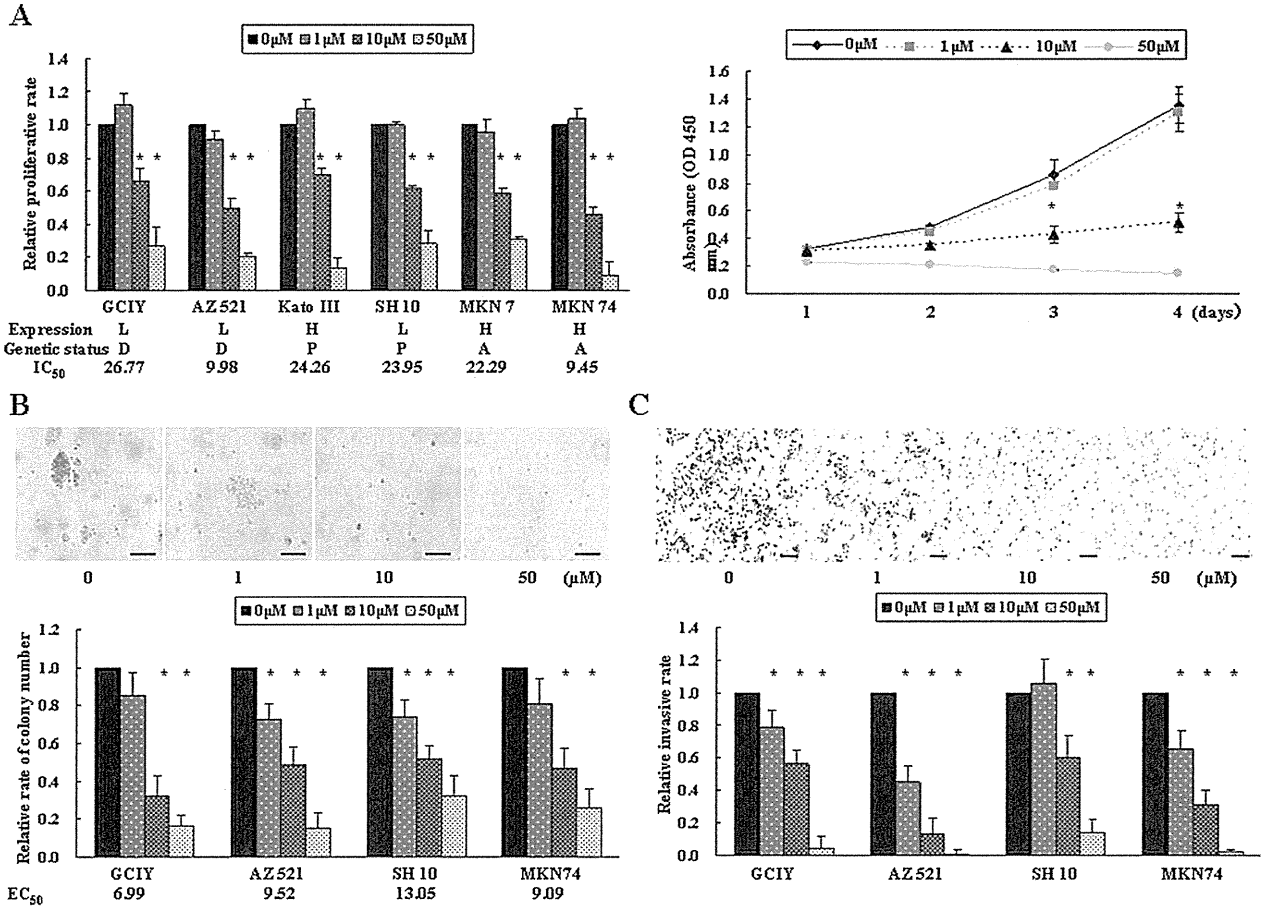

Figure 4 Anticancer activity of PRL-3 inhibitor. (A) Proliferation assay after treatment with PRL-3 inhibitor at concentrations ranging from 0 to 50 μmol/L against 6 cell lines with different PRL-3 expression and genetic status. With cells treated with chemical solution alone (0 μmol/L PRL-3 inhibitor) 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.

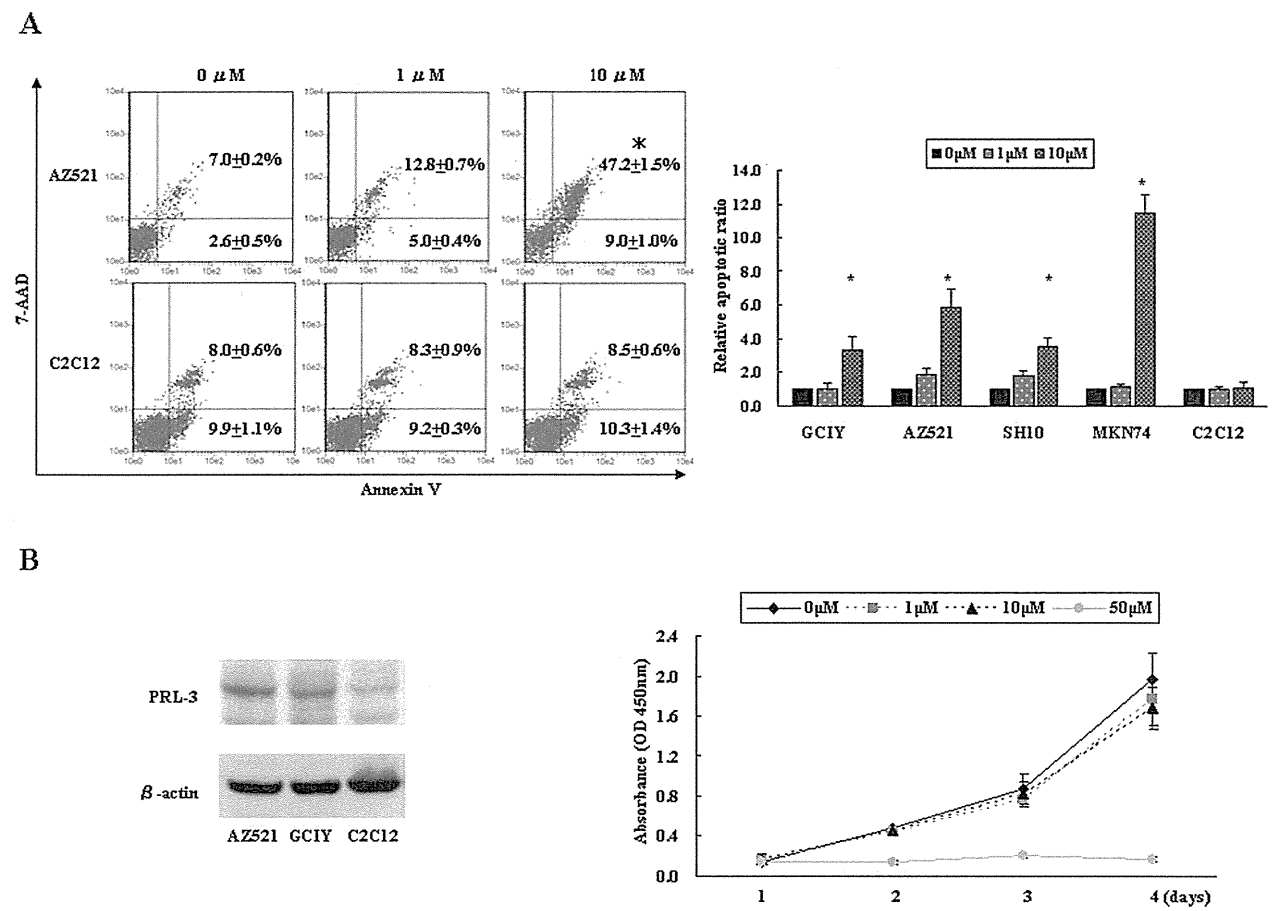

Figure 5 PRL-3 inhibitor-mediated apoptosis. (A) Apoptosis assay was performed 72 hours after treatment with PRL-3 inhibitor (0 to 10 μmol/L). Representative figures of apoptosis assay on AZ521 and C2C12 cells were shown in left panel, and the percentage and SD of early apoptosis (bottom right quadrant) 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