High PRL-3 expression in human gastric cancer is a marker of metastasis and grades of malignancies: an in situ hybridization study

Miskad, Upik Anderiani ; Semba, Shuho ; Kato, Hirotaka ; Matsukawa, Yasuko ; Kodama, Yoshinori ; Mizuuchi, Eri ; Maeda, Naoko ; Yanagihara…

(Citation)
Virchows Archiv,450(3):303-310

(Issue Date)
2007-03

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/90000586

ORIGINAL ARTICLE

High *PRL-3* expression in human gastric cancer is a marker of metastasis and grades of malignancies: An *in situ* hybridization study

Upik Anderiani Miskad ・ Shuho Semba ・ Hirotaka Kato ・ Yasuko Matsukawa ・ Yoshinori Kodama ・ Eri Mizuuchi ・ Naoko Maeda ・ Kazuyoshi Yanagihara ・ Hiroshi Yokozaki

U. A. Miskad ・ S. Semba ・ H. Kato ・ Y. Matsukawa ・ Y. Kodama ・ E. Mizuuchi ・ N. Maeda ・ H. Yokozaki
Division of Surgical Pathology, Department of Biomedical Informatics, Kobe University Graduate School of Medicine, Kobe, 650-0017, Japan.

K. Yanagihara
Central Animal Laboratory, National Cancer Center Research Institute, Tokyo, 104-0045, Japan.

Running title: *PRL-3* expression in human gastric carcinomas.

Address for reprints: Hiroshi Yokozaki, M.D., Ph.D., Division of Surgical Pathology, Department of Biomedical Informatics, Kobe University Graduate School of Medicine
7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017 (Japan)
Tel. (81) 78-382-5460; Fax (81) 78-382-5479; e-mail: hyoko@med.kobe-u.ac.jp

Abstract *Phosphatase of regenerating liver (PRL)-3*, encoding a 22-kD low molecular weight tyrosine phosphatase, has been reported to be associated with metastasis of colorectal carcinoma. We assessed the levels of *PRL-3* mRNA expression to know whether its up-regulation was involved in progression and metastasis of gastric carcinoma. Levels of *PRL-3* expression in 94 human gastric adenocarcinomas and 54 matched lymph node metastases were detected by *in situ* hybridization and compared with clinicopathological characteristics including prognosis. High *PRL-3* expression was detected in 36.2% of primary gastric carcinoma (with nodal metastasis, 55.6%; without nodal metastasis, 10%; $P < 0.001$) and in 74.1% of lymph node metastases. The incidence of high *PRL-3* expression in lymph node metastasis was significantly higher than in primary tumors ($P < 0.044$). Moreover, high expression of *PRL-3* was closely associated with tumor size, lymphatic invasion, venous invasion, extent of lymph node metastasis and tumor stage. These results suggest that high *PRL-3* expression may participate in the progression and metastasis of gastric carcinoma. *PRL-3* might be a novel molecular marker for aggressive gastric cancer.

Keywords *PRL-3* · gastric carcinoma · *in situ* hybridization · lymph node metastasis

Introduction

Gastric cancer incidence and mortality has fallen dramatically over the last 50 years in many regions, but remains the second most common cancer worldwide [1, 23]. Metastasis of gastric cancer to the lymph nodes, peritoneum and/or other organs is often responsible for highest mortality rate because the primary tumors can usually be surgically removed [4, 9]. Involvement of regional lymph nodes is important prognostic factor for gastric carcinoma: *i.e.* if lymph nodes are found to be negative for metastasis by thorough pathologic examination, over 50% of the patients may be expected to survive for 5 years, otherwise the survival rate drops to less than 10 % [4, 8]. Mechanisms of gastric cancer metastasis are not fully clarified because of the involvement of multiple steps with the accumulation of altered expression of lots of different genes [29]. Although many molecular factors participating in metastasis have been identified from studies that use microarray expression profiling [9], much remains to be learned about the process to provide a new therapeutic target for these metastatic lesions.

Protein tyrosine phosphatases (PTPs) play fundamental roles in regulating diverse proteins that participate essentially in every aspect of cellular physiological and pathogenic processes [33]. Phosphatase of regenerating liver (PRL)-1, -2 and -3 represent a novel class of PTPs superfamily members in possessing a unique COOH-terminal prenylation motif with PTP active site signature sequence CX_5R [5, 30]. PRL phosphatases were found to be associated with the early endosome and plasma membrane in their prenylated state, while nuclear localization of these enzymes may occur in the absence of prenylation [31]. All are proteins of about 20-kD and share 75% amino acid sequence similarity [6, 30]. *PRL-1* was originally identified as an immediate early gene whose expression was induced in mitogen-stimulated cells and regenerating liver [16]. Overexpression of PRL-1 and PRL-2 transformed mouse fibroblasts and hamster pancreatic epithelial cells in culture and promoted

tumor growth in nude mice, suggesting that it may participate in the tumorigenesis [5, 6].

PRL-3 has been found to enhance growth of human embryonic kidney fibroblasts [14]. Among normal human adult tissues, it is expressed predominantly in the heart and skeletal muscle cells with lower expression in the pancreas, and this expression pattern is distinct from the wider expression of PRL-1 and PRL-2 [14]. Gene expression profiling using serial analysis of gene expression (SAGE) revealed that among 144 up-regulated genes detected in liver samples of metastatic colorectal cancer, *PRL-3* was the only gene consistently overexpressed in all cancer metastasis cases examined with lower levels in non-metastatic tumors and normal colorectal epithelium [24]. Overexpression of *PRL-3* was reported to be associated with human colorectal cancer metastasis and *PRL-3* was apparently expressed in colorectal cancer metastases to any organ [2, 11]. In addition, stable transfection of wild-type *PRL-3* in non or less metastatic CHO or mouse melanoma B16 cells has been reported to induce the acquisition of metastasis-associated phenotypes and to confer metastatic ability, whereas a catalytically inactive PRL-3 mutant greatly reduced the effect on promoting cell migration [28, 32]. Moreover, specific anti-sense oligodeoxynucleotide and phosphatase inhibitors could reverse the acquired higher migratory ability with exogenously overexpressed PRL-3 [28]. Conversely, transient down-regulation of *PRL-3* expression in DLD-1 human colon cancer cells with a specific small interfering RNA abrogated cellular motility *in vitro* and *in vivo* hepatic colonization after infection into the nude mice spleen suggesting the possible contribution of PRL-3 to the establishment of liver metastasis, especially at the step in which cancer cells leave the circulation to extravasate into the liver tissue [11]. Moreover, stable knockdown of PRL-3 by artificial microRNA in SGC7901 human gastric cancer cells effectively suppressed the growth of experimental peritoneal metastases in nude mice independent of cellular proliferation [13]. These experimental evidences support the significant role of overexpressed and catalitically active PRL-3 in cancer metastasis.

We previously conducted an immunohistochemical analysis of human gastric carcinomas using anti-PRL-3 antibody and reported that high expression of PRL-3 immunoreactivity was frequently detected in those with lymph node metastasis [15]. However, we could not eliminate the possibility of cross-reaction of the antibody to PRL-1 and/or PRL-2. To clarify this, in the present study, we investigated the levels of *PRL-3* mRNA expression by highly specific *in situ* hybridization (ISH) in the primary gastric carcinoma tissues as well as corresponding non-neoplastic mucosa and matched regional lymph node metastases and correlated with clinicopathological features and prognosis of the patients.

Materials and methods

Tissue samples

A total of 152 formalin-fixed and paraffin-embedded surgical specimens of primary human gastric carcinomas and lymph node metastases were collected from the Department of Pathology, Kobe University Hospital. They consisted of 54 cases of primary tumors along with matched lymph node metastases and 40 cases of primary tumors without lymph node metastasis. Four cases with liver metastasis and peritoneal metastasis were also available. They consisted of 68 men and 26 women with an age range of 34-87 years and mean 66.82 years. Informed consent was obtained from all patients. We classified gastric carcinomas according to the criteria of the Japanese Classification of Gastric Carcinoma [10].

Oligonucleotide probe and ISH

The expression levels of PRL-3 in tissue sections were analyzed using ISH as described

elsewhere with some modifications [3, 11]. Specific antisense oligonucleotide DNA probe was designed complementary to the mRNA transcript of *PRL-3* gene, based on published reports of the cDNA sequences. The sequence of probe was 5' GTTGATGGCTCCGCGGCG-Brigati Tail-3' with 72.2 % GC content. The specificity of the oligonucleotide sequences was initially determined by a GenBank/European Molecular Biology Laboratory database search using the Genetics Computer Group sequence analysis program (Genetics Computer Group, Madison, WI) based on the FastA algorithm [18] that showed 100% homology with the target gene and minimal homology with non-specific mammalian gene sequences. DNA probe was synthesized with six biotin molecules (hyperbiotinylated) at the 3' end via direct coupling using standard phosphormidine chemistry (Invitrogen, Carlsbad, CA) [17].

ISH was carried out using the Microprobe manual staining system (Fisher Scientific, Pittsburgh, PA). Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on Silane-coated ProbeOn slides (Fisher Scientific). The slides were placed in the Microprobe slide holder, dewaxed, dehydrated and followed by enzymatic digestion with pepsin (Dako, Carpinteria, CA). Hybridization of the probe was carried out for 90 min at 45°C, and the samples were then washed three times with 2x SSC for 2 min at 45°C. The samples were incubated for 30 min in alkaline phosphatase-labeled avidin (Biomeda, Foster City, CA) at 45°C, briefly rinsed in 50 mM Tris-HCl (pH 7.6), rinsed for 1 min with alkaline phosphatase enhancer, and incubated for 20 min with the chromogen substrate FastRed (Biomeda) at 45°C. Hybridization of the samples with a biotinylated poly$(dT)_{20}$ oligonucleotide was used to verify the integrity of mRNA in each sample. To analyze the specificity of the hybridization signal, we performed the following controls: (1) RNase pretreatment of tissue sections, (2) competitive assay with sense probe, (3) treatment of the samples in the absence of the biotinylated probe to control endogenous alkaline phosphatase and (4) use of chromogen in the absence of any oligonucleotide probes. A markedly decreased or absent signal was obtained

under all of these conditions. For evaluation of staining, normal gastric epithelium, smooth muscle cells of the vessels and infiltrated inflammatory cells were used as internal controls. Staining in over 25% cancer cells obviously exceeding the internal controls was regarded as "high expression" and negative or no increase of staining in cancer cells compared with them was judged as "low/none expression". The grades of *PRL-3* expression were determined by three independent observers (U.A.M., S.S., H.K.) unaware of the clinical and histological diagnoses. All of the sections were scored twice to confirm the reproducibility of the results and any discordant result was settled by using a conference microscope.

Immunohistochemistry

We determined Ki-67 labeling index and apoptotic body index immunohistochemically as described elsewhere [25], using monoclonal antibody against Ki-67 antigen (Dako, Japan; 1:100 dilution) and polyclonal antibody against single stranded DNA (ssDNA, Dako; 1:100 dilution), respectively. The Ki-67 labeling index and apoptotic body index were calculated by counting the number of positive cells (%) in a total of 1000 or more tumor cells observed in 10 representative high-power fields. Values were presented as the mean ± SD (standard deviation).

Statistical methods

The relationships between the results of the ISH study and clinicopathological variables were tested by χ^2 test and Mann-Whitney *U* test. Survival analysis was computed by the Kaplan-Meier method and compared by log-rank test. A *P* value less than 0.05 was regarded as statistically significant.

Results

We examined the expression and localization of *PRL-3* in 148 cases of gastric carcinoma and lymph node metastasis by using ISH method that facilitated the study of paraffin-embedded specimens. We confirmed that all samples had intense histochemical reaction with poly$(dT)_{20}$ probe, indicating that the mRNA was well preserved (Fig. 1A). Negative to weak *PRL-3* expression was detected in non-neoplastic gastric mucosa including foveolar epithelia, fundic and pyloric glandular cells. Weak to moderate expression was also detected in smooth muscle cells of the vessels and in some inflammatory cells, such us lymphocytes and plasma cells. However, in the tumor cells, *PRL-3* expression was varied and observed heterogeneously among the cases. Prominent hybridization indicating high *PRL-3* expression was detected in 34 (36.2%) of 94 primary gastric carcinoma cases examined. Representative results of *PRL-3* ISH staining are shown in Fig. 1B. We then analyzed the relationship between expression of *PRL-3* and clinicopathological parameters of gastric carcinoma. Results are summarized in Table 1. High expression of *PRL-3* was significantly associated with gender ($P = 0.010$), tumor size ($P = 0.017$) and stage of tumor ($P = 0.008$). Also, a similar correlation was observed with cancer progression, such as lymphatic invasion ($P < 0.001$), venous invasion ($P = 0.012$) and extent of lymph node metastasis ($P < 0.001$). Although not statistically significant, the incidence of *PRL-3* expression tended to be associated with depth of tumor invasion ($P = 0.051$). Additionally, high expression of *PRL-3* was also associated with the high level of Ki-67 labeling index ($P = 0.032$) and low level of apoptotic bodies ($P = 0.030$). There was no significant correlation between *PRL-3* expression and age or histological type.

As shown in Table 2, among primary gastric carcinomas, the incidence of cases with high *PRL-3* expression was significantly frequent in carcinomas with nodal metastasis (30

[55.6%] of 54 cases) than in those without nodal metastasis (4 [10%] of 40 cases) ($P < 0.001$). Simultaneously, we also investigated the expression of *PRL-3* in regional lymph node metastases. Interestingly, out of 54 cases with lymph node metastases, high *PRL-3* expression was detected in 40 cases (74.1%). The frequency of high *PRL-3* expression was increased in matched lymph node metastases in comparison with primary gastric carcinomas ($P = 0.044$). Moreover, high *PRL-3* expression was also detected in other metastatic sites, such as liver and peritoneum.

Finally, we checked the survival rate of gastric cancer patients after surgery. The cases with high expression of *PRL-3* showed a tendency have a worse prognosis than the cases with low/none expression of *PRL-3*, although it was not statistically significant ($P = 0.165$) (Fig. 2).

Discussion

Histochemical evaluation of PRL-3 expression in human cancer tissues has been reported mainly on colorectal carcinomas. Bardelli *et al*. developed ISH methods for the study of paraffin-embedded colorectal cancer sections and reported that *PRL-3* expression was elevated in nearly all metastatic lesions derived from colorectal cancers, regardless of the site of metastasis, while they observed little or no *PRL-3* mRNA expression in normal colon, non-metastatic primary cancers or metastatic lesions derived from cancers of pancreas, stomach or esophagus [2]. On the other hand, Kato *et al*., also using ISH methods on paraffin-embedded specimens, detected *PRL-3* expression in primary colorectal cancers of which the frequency of up-regulated *PRL-3* expressions in cases with liver or lung metastasis was statistically higher than that in cases without either type of metastasis [11]. Peng *et al*. prepared a specific monoclonal antibody against human PRL-3 and detected its

immunoreactivity in 7% of normal colorectal epithelia, 23.9% of primary colorectal cancers, 53.7% of metastatic lymph nodes and 66.7% of liver metastases [19, 20]. Previously, we conducted immunohistochemical analysis of PRL-3 on human gastric cancer tissues using commercial antibody and reported that overexpression of PRL-3 immunoreactivity in primary gastric carcinoma was closely associated with lymphatic invasion, extent of lymph node metastasis and tumor stage [15]. However, as PRL-3 shares 78% and 75% amino acid sequence similarity with PRL-1 and PRL-2, respectively [12], we could not exclude the possibility of the cross reaction of the anti-PRL-3 antibody used in the previous study to other PRL family phosphatases.

The present ISH study, highly specific for the detection of *PRL-3* mRNA, on the same gastric cancer specimens as was investigated in the previous immunohistochemical analysis [15] confirmed the *PRL-3* expression in primary as well as metastatic gastric cancer cells. Interestingly, high level of *PRL-3* expression in primary gastric carcinoma tissue was closely associated not only with clinicopathological factors characterized by immunohistochemistry (i. e., lymphatic invasion, extent of lymph node metastasis and tumor stage) [15], but also with tumor size, venous invasion and either higher levels of Ki-67 labeling index as well as lower levels of apoptotic body index. Moreover, high expression of *PRL-3* was significantly frequent in primary gastric carcinoma with nodal metastasis than those without nodal metastasis that was also observed in the previous study [15]. Consistent with the elevated expression of *PRL-3* in colorectal cancer metastasis [2, 11, 20, 24], the frequency of high *PRL-3* expression was significantly increased in matched lymph node metastases than in primary gastric tumors. The growth-promoting effect of ectopically overexpressed PRL-3 in HEK293 human embryonic kidney epithelial cells [14] and mouse B16 melanoma cells [28] have been reported. Although enhanced cell cycle progression and down-regulation of p21cip1/waf1, a cyclin-dependent kinase inhibitor, by PRL-1 or PRL-2

typrosine phosphatases was demonstrated in D27 hamster pancreatic ductal epithelial cells [27], effect of overexpressed PRL-3 on the cell cycle or apoptosis has not been elucidated yet. These findings suggest that high *PRL-3* expression might not only have important roles in invasion and metastasis but also play some role in cellular proliferation and/or growth of human gastric cancer.

Elevated PRL-3 protein or mRNA expression in the primary tumor has been reported to associate with prognosis, including disease free- as well as overall-survival, of colorectal and breast cancer patients [11, 20, 22, 26]. The present study demonstrated that patients with high expression of *PRL-3* in their primary gastric cancer tissue tended to have worse prognosis than those with low or negative PRL-3 expression, while we could not obtain a significant statistical difference.

These results overall suggest that overexpression of PRL-3 may contribute to the progression and metastasis of human gastric carcinoma. Histochemical detection of PRL-3 expression may serve as a good marker for the prediction of grade of malignancy. Inhibition of prenylation and/or inactivating the catalytic function of PRL-3 phosphatase could block or reduce the cellular properties of invasion and metastasis [32]. Therefore, elucidation of precise biological function of PRL-3 may provide an attractive molecular target for gastric cancer control. Although it has been reported that PRL-3 interacted with integlin $\alpha 1$ [21] and regulated Rho family GTPases [7], further investigations will be required to clarify the exact pathway(s) of PRL-3 driven cancer metastasis.

Acknowledgement This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare of Japan (14-7). It is also supported in part by Grants-in-Aid for Scientific Research (B) (14370070) and Exploratory Research (18659096) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. Grant from

the Terry Fox Run Foundation for Cancer Research was also indebted. The authors thank to Prof. Hiroki Kuniyasu (Department of Molecular Pathology, Nara Medical University) for valuable discussion and technical assistance.

References

1. Alberts SR, Cervantes A, van de Velde CJ (2003) Gastric cancer: epidemiology, pathology and treatment. Ann Oncol 14 Suppl 2:ii31-36
2. Bardelli A, Saha S, Sager JA, Romans KE, Xin B, Markowitz SD, Lengauer C, Velculescu VE, Kinzler KW, Vogelstein B (2003) PRL-3 expression in metastatic cancers. Clin Cancer Res 9:5607-5615
3. Bucana CD, Radinsky R, Dong Z, Sanchez R, Brigati DJ, Fidler IJ (1993) A rapid colorimetric *in situ* mRNA hybridization technique using hyperbiotinylated oligonucleotide probes for analysis of mdr1 in mouse colon carcinoma cells. J Histochem Cytochem 41:499-506
4. Buchholtz TW, Welch CE, Malt RA (1978) Clinical correlates of resectability and survival in gastric carcinoma. Ann Surg 188:711-715
5. Cates CA, Michael RL, Stayrook KR, Harvey KA, Burke YD, Randall SK, Crowell PL, Crowell DN (1996) Prenylation of oncogenic human PTP_{CAAX} protein tyrosine phosphatases. Cancer Lett 110:49-55
6. Diamond RH, Cressman DE, Laz TM, Abrams CS, Taub R (1994) PRL-1, a unique nuclear protein tyrosine phosphatase, affects cell growth. Mol Cell Biol 14:3752-3762
7. Fiordalisi JJ, Keller PJ, Cox AD (2006) PRL tyrosine phosphatases regulate rho family GTPases to promote invasion and motility. Cancer Res 66:3153-3161
8. Hawley PR, Westerholm P, Morson BC (1970) Pathology and prognosis of carcinoma of

the stomach. Br J Surg 57:877-883

9. Hippo Y, Yashiro M, Ishii M, Taniguchi H, Tsutsumi S, Hirakawa K, Kodama T, Aburatani H (2001) Differential gene expression profiles of scirrhous gastric cancer cells with high metastatic potential to peritoneum or lymph nodes. Cancer Res 61:889-895
10. Japanese Gastric Cancer Association (1998) Japanese Classification of Gastric Carcinoma -2nd English Edition -. Gastric Cancer 1:10-24
11. Kato H, Semba S, Miskad UA, Seo Y, Kasuga M, Yokozaki H (2004) High expression of *PRL-3* promotes cancer cell motility and liver metastasis in human colorectal cancer: a predictive molecular marker of metachronous liver and lung metastases. Clin Cancer Res 10:7318-7328
12. Kim KA, Song JS, Jee J, Sheen MR, Lee C, Lee TG, Ro S, Cho JM, Lee W, Yamazaki T, Jeon YH, Cheong C (2004) Structure of human PRL-3, the phosphatase associated with cancer metastasis. FEBS Lett 565:181-187
13. Li Z, Zhan W, Wang Z, Zhu B, He Y, Peng J, Cai S, Ma J (2006) Inhibition of PRL-3 gene expression in gastric cancer cell line SGC7901 via microRNA suppressed reduces peritoneal metastasis. Biochem Biophys Res Commun 348:229-237
14. Matter WF, Estridge T, Zhang C, Belagaje R, Stancato L, Dixon J, Johnson B, Bloem L, Pickard T, Donaghue M, Acton S, Jeyaseelan R, Kadambi V, Vlahos CJ (2001) Role of PRL-3, a human muscle-specific tyrosine phosphatase, in angiotensin-II signaling. Biochem Biophys Res Commun 283:1061-1068
15. Miskad UA, Semba S, Kato H, Yokozaki H (2004) Expression of PRL-3 phosphatase in human gastric carcinomas: close correlation with invasion and metastasis. Pathobiology 71:176-184
16. Mohn KL, Laz TM, Hsu JC, Melby AE, Bravo R, Taub R (1991) The immediate-early growth response in regenerating liver and insulin-stimulated H-35 cells: comparison with

serum-stimulated 3T3 cells and identification of 41 novel immediate-early genes. Mol Cell Biol 11:381-390

17. Park CS, Manahan LJ, Brigati DJ (1991) Automated molecular pathology: one hour *in situ* hybridization. J Histotechnol 14:219-229
18. Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci U S A 85:2444-2448
19. Peng L, Li Y, Meng L, Shou C (2004) Preparation and characterization of monoclonal antibody against protein tyrosine phosphatase PRL-3. Hybrid Hybridomics 23:23-27
20. Peng L, Ning J, Meng L, Shou C (2004) The association of the expression level of protein tyrosine phosphatase PRL-3 protein with liver metastasis and prognosis of patients with colorectal cancer. J Cancer Res Clin Oncol 130:521-526
21. Peng L, Jin G, Wang L, Guo J, Meng L, Shou C (2006) Identification of integrin alpha1 as an interacting protein of protein tyrosine phosphatase PRL-3. Biochem Biophys Res Commun 342:179-183
22. Radke I, Gotte M, Kersting C, Mattsson B, Kiesel L, Wulfing P (2006) Expression and prognostic impact of the protein tyrosine phosphatases PRL-1, PRL-2, and PRL-3 in breast cancer. Br J Cancer 95:347-354
23. Roder DM (2002) The epidemiology of gastric cancer. Gastric Cancer 5 Suppl 1:5-11
24. Saha S, Bardelli A, Buckhaults P, Velculescu VE, Rago C, St Croix B, Romans KE, Choti MA, Lengauer C, Kinzler KW, Vogelstein B (2001) A phosphatase associated with metastasis of colorectal cancer. Science 294:1343-1346
25. Semba S, Itoh N, Ito M, Youssef EM, Harada M, Moriya T, Kimura W, Yamakawa M (2002) Down-regulation of PIK3CG, a catalytic subunit of phosphatidylinositol 3-OH kinase, by CpG hypermethylation in human colorectal carcinoma. Clin Cancer Res 8:3824-3831

26. Wang L, Peng L, Dong B, Kong L, Meng L, Yan L, Xie Y, Shou C (2006) Overexpression of phosphatase of regenerating liver-3 in breast cancer: association with a poor clinical outcome. Ann Oncol 17:1517-1522

27. Werner SR, Lee PA, DeCamp MW, Crowell DN, Randall SK, Crowell PL (2003) Enhanced cell cycle progression and down regulation of $p21^{Cip1/Waf1}$ by PRL tyrosine phosphatases. Cancer Lett 202:201-211

28. Wu X, Zeng H, Zhang X, Zhao Y, Sha H, Ge X, Zhang M, Gao X, Xu Q (2004) Phosphatase of regenerating liver-3 promotes motility and metastasis of mouse melanoma cells. Am J Pathol 164:2039-2054

29. Yokozaki H, Yasui W, Tahara E (2001) Genetic and epigenetic changes in stomach cancer. Int Rev Cytol 204:49-95

30. Zeng Q, Hong W, Tan YH (1998) Mouse PRL-2 and PRL-3, two potentially prenylated protein tyrosine phosphatases homologous to PRL-1. Biochem Biophys Res Commun 244:421-427

31. Zeng Q, Si X, Horstmann H, Xu Y, Hong W, Pallen CJ (2000) Prenylation-dependent association of protein-tyrosine phosphatases PRL-1, -2, and -3 with the plasma membrane and the early endosome. J Biol Chem 275:21444-21452

32. Zeng Q, Dong JM, Guo K, Li J, Tan HX, Koh V, Pallen CJ, Manser E, Hong W (2003) PRL-3 and PRL-1 promote cell migration, invasion, and metastasis. Cancer Res 63:2716-2722

33. Zhang ZY, Zhou B, Xie L (2002) Modulation of protein kinase signaling by protein phosphatases and inhibitors. Pharmacol Ther 93:307-317

Fiugre legends

Fig. 1 *In situ* hybridization (ISH) analysis of *PRL-3* mRNA expression in human gastric cancer tissues. **A** Hybridization with a biotinylated poly$(dT)_{20}$ oligonucleotide probe (*a*) is performed to verify the integrity of mRNA. A positive ISH reaction of *PRL-3* with the specific antisense probe (*b*) in this assay provides red staining in the cytoplasm. Negative chromogen reaction is confirmed with the sense oligonuculeotide probe (c) with the same specimen as (*a*) or (*b*). Bars: 100 μm. **B** Representative results of *PRL-3* ISH in primary gastric cancer (*d*), metastatic cancer in regional lymph node of the stomach (*e*) and peritoneal metastasis (*f*). Consecutive sections stained with hematoxylin and eosin are displayed in (*g*), (*h*) and (*i*). High levels of *PRL-3* expression is detected in cancer cells, while non-neoplastic gastric mucosa ((*d*), (*g*); arrow head) demonstrated negative to low expression. Bars: 200 μm.

Fig. 2 Kaplan-Meier analysis of gastric cancers. Cancer-related suvival rates of patients whose gastric cancer tissue showed high (solid line) or low/none (hatched line) expression of *PRL-3* mRNA assessed by ISH. Patients with gastric cancers expressing high levels of *PRL-3* showed tendency to have much worse prognosis than those with primary gastric tumors exhibiting low/none *PRL-3* expression ($P = 0.165$).

Table 1 Expression of *PRL-3* in human gastric carcinomas and its correlation with clinicopathological parameters, Ki-67 labeling index and apoptotic body index

	Number of cases	*PRL-3* expression [a] High n (%)	Low / none n (%)	*P* value [d]
Total	94	34 (36.2)	60 (63.8)	
Sex				
Male	68	30 (44.1)	38 (55.9)	0.010*
Female	26	4 (15.4)	22 (84.6)	
Age				
≤ 65	38	13 (34.2)	25 (65.8)	0.745
> 65	56	21 (37.5)	35 (62.5)	
Histology [b]				
Well	53	23 (43.4)	30 (56.6)	0.097
Poorly	41	11 (26.8)	30 (73.2)	
Tumor size [c]				
≤ 40mm	43	10 (23.3)	33 (76.7)	0.017*
> 40mm	51	24 (47.1)	27 (52.9)	
Lymphatic invasion				
ly (-)	33	4 (12.1)	29 (87.9)	< 0.001**
ly (+)	61	30 (49.2)	31 (50.8)	
Venous invasion				
v (-)	41	9 (22.9)	32 (78.1)	0.012*
v (+)	53	25 (47.2)	28 (52.8)	
Depth of invasion [b]				
T1	33	6 (18.2)	27 (81.8)	0.051
T2	23	12 (52.2)	11 (47.8)	
T3	28	12 (42.9)	16 (57.1)	
T4	10	4 (40)	6 (60)	
Extent of lymph node metastasis [b]				
N0	40	4 (10)	36 (90)	< 0.001**
N1	27	16 (59.3)	11 (40.7)	
N2	18	8 (44.4)	10 (55.6)	
N3	9	6 (66.7)	3 (33.3)	
Tumor stage [b]				
I	38	6 (15.8)	32 (84.2)	0.008*
II	14	6 (42.9)	8 (57.1)	
III	28	15 (53.6)	13 (46.4)	
IV	14	7 (50)	7 (50)	
Ki-67 labeling index (mean ± SD) [e]		45.96 ± 9.82	40.49 ± 11	0.032*
Apoptotic body index (mean ± SD) [e]		13.07 ± 5.05	17.31 ± 8.2	0.030*

[a] The levels of *PRL-3* expression were determined by ISH. Grades of *PRL-3* staining were

classified as high, low or negative as described in the text.

[b] According to the criteria of the Japanese Classification of Gastric Carcinoma [10]. Well, well-differentiated type including papillary adenocarcinoma, well-differentiated tubular adenocarcinoma and moderately differentiated tubular adenocarcinoma; poorly, poorly differentiated type including solid-type poorly differentiated adenocarcinoma, non-solid type poorly differentiated adenocarcinoma, signet-ring cell carcinoma and mucinous adenocarcinoma. Depth of carcinoma invasion and extent of lymph node metastasis were classified into T1-T4 and N0-N3, respectively

[c] Tumor size was divided into two groups according to the maximum diameter.

[d] Statistical analyses were performed by χ^2 test and Mann-Whitney *U* test. A *P* value less than 0.05 was regarded as statistically significant. $*P < 0.05$, $**P < 0.001$.

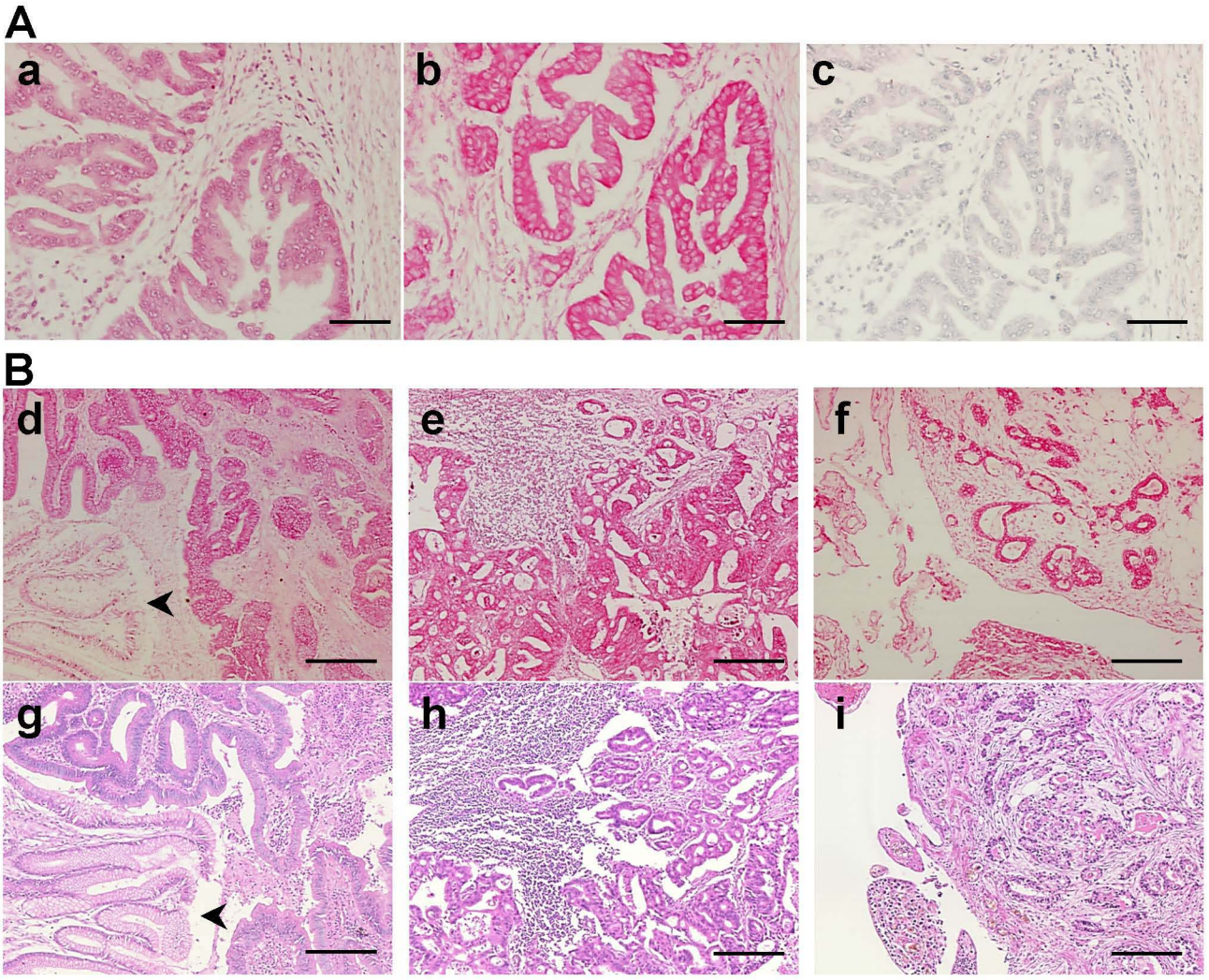
[e] Ki-67 labeling index and apoptotic body index were determined by counting the number of positive cells (%) in a total of 1000 or more tumor cells observed in 10 representative high-power fields (x400). SD: standard deviation.

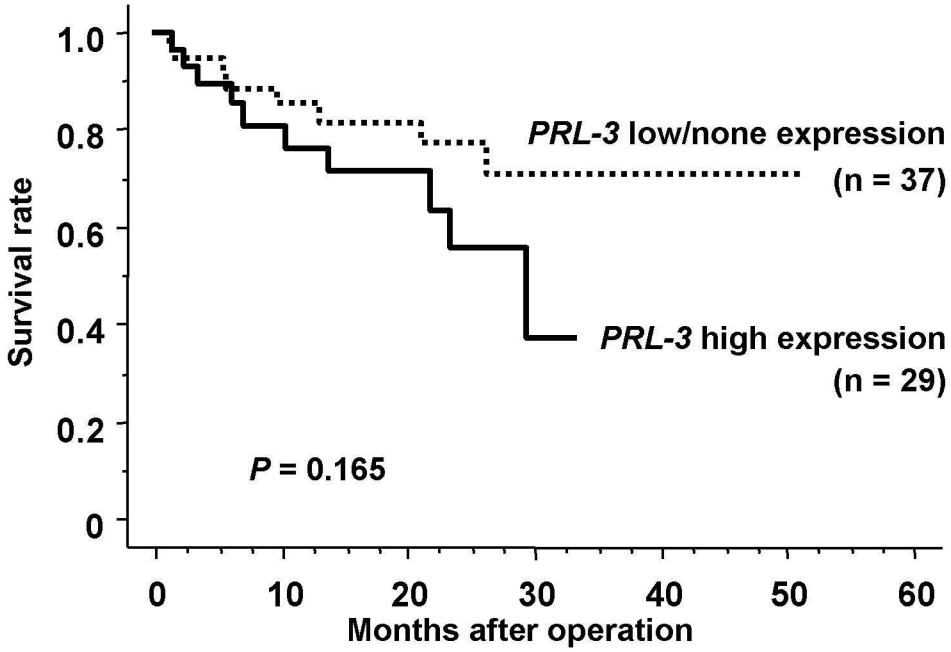
Table 2 Expression of *PRL-3* in human gastric carcinomas and lymph node metastasis

	Number of cases	*PRL-3* expression [a] High n (%)	Low / none n (%)	*P* value [b]
Primary gastric carcinomas	94			
with nodal metastases	54	30 (55.6)	24 (44.4)	< 0.001**
without nodal metastasis	40	4 (10)	36 (90)	
Gastric carcinomas with nodal metastases				
Primary gastric carcinomas	54	30 (55.6)	24 (44.4)	0.044*
Matched lymph node metastases	54	40 (74.1)	14 (25.9)	

[a] The levels of *PRL-3* expression were determined by ISH. Grades of *PRL-3* staining were classified as high, low or negative as described in the text.

[b] Statistical analysis was performed by χ^2 test. A P value less than 0.05 was regarded as statistically significant. *$P < 0.05$, **$P < 0.001$.


A
a
b
c
B
d
e
f
g
h
i


1.0
0.8
0.6
0.4
0.2
0
Survival rate
PRL-3 low/none expression
(n = 37)
PRL-3 high expression
(n = 29)
P = 0.165
0
10
20
30
40
50
60
Months after operation