Expression level of Pre B cell leukemia homeobox 2 correlates with poor prognosis of gastric adenocarcinoma and esophageal squamous cell carcinoma

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Abstract. Pre B cell leukemia homeobox 2 (PBX2), a member of PBX family, acts as a co-factor of homeobox proteins to regulate proliferation and differentiation of tumor cells. Our recent study revealed prognostic significance of PBX2 expression in non-small cell lung carcinoma. The significance of PBX2 expression was examined in cases with gastric cancer (GC) and esophageal squamous cell carcinoma (ESCC), and the role of PBX2 in tumor behavior was evaluated in GC and ESCC cell lines of knocked-down PBX2 expression. Expression level of PBX2 was immunohistochemically examined in 94 patients of GC and 64 patients of ESCC. Staining intensity for PBX2 was categorized as equal to or stronger (level 1) and weaker (level 2) than that of endothelial cells. Cases with level 1 expression in more than 20% of tumor were defined as high and others low expression. Patients with low PBX2 expression showed a better prognosis than those with high expression in both GC and ESCC. Multivariate analysis revealed PBX2 expression to be an independent prognosticator for both GC and ESCC. Knocked-down expression of PBX2 in GC and ESCC cell lines resulted in decrease of *in vitro* colony formation and *in vivo* tumorigenic activities, but proliferative and invasive activities did not change. Under serum depletion, apoptotic cell proportion was higher in PBX2 knocked-down cells than in control cells. The knock-down of PBX2 reduced Bcl-2 expression. Taken together, the high expression level of PBX2 was an independent negative prognosticator for both GC and ESCC, and PBX2 might promote tumor growth through suppression of apoptosis.

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Introduction

Pre B cell leukemia homeobox (PBX) family belongs to the superfamily of homeodomain-containing proteins, among which homeobox (HOX) proteins are most intensively investigated. HOX proteins act as transcription factors in cooperation with other regulatory proteins, including PBX2, and are involved in the control of developmental processes and cell differentiation (1,2). Aberrant overexpression of HOX genes has been reported in various kinds of cancers, such as oral squamous cell carcinoma, non-small cell lung carcinoma (NSCLC), ovarian carcinoma, esophageal squamous cell carcinoma (ESCC), gastric carcinoma (GC), hepatocellular carcinoma, prostate cancer, bladder cancer, and cervical cancer, together with acute lymphoblastic leukemia (3-12). Overexpression of HOX proteins is correlated with poor prognosis in some cancers, such as acute myeloid leukemia (13,14). In contrast to other HOX proteins, PBX family proteins have not been studied in detail. The interference of PBX binding to HOX proteins yielded apoptosis and reduced growth activity of tumors *in vivo*, indicating that the interaction of PBX to HOX appears to be crucial in tumor behavior (15-17).

GC and ESCC are the most common cancers not only in Asia but also in the world. They are highly aggressive carcinomas with poor outcome: annually over 1 million people die of these cancers worldwide (18-20). In Japan and Southern China, 5-year survival rate of GC and ESCC patients is less than 20%, due to the occasional occurrence of systemic metastasis within a few years even after curative resection (21-23). For improvement of prognosis of these patients, it is essential to understand molecular mechanisms underlying

aggressiveness of GC and ESCC. Our recent study revealed that the NSCLC with high level of PBX2 expression showed a poorer prognosis than that with low expression (24). In the present study, the expression level of PBX2 proteins was immunohistochemically examined in 94 patients with GC and 64 with ESCC, who underwent curative surgery, and its correlation with clinicopathological features and prognosis of patients was analyzed. To evaluate the role of PBX2 in tumor behavior, the effects of knock-down of this gene on colony formation, apoptosis, proliferation and invasive activities in GC and ESCC cell lines were estimated.

Patients and methods

Patients. Gastric cancer. A total of 94 patients with histologically proven GC were analyzed: they underwent curative resection at the Department of Surgery, East Hospital of Tongji University (Shanghai, China) between 1998 and 2002. Curative resection is defined as the complete resection of gastric lesions, with no remaining tumorous lesions at the postoperative histological examinations. There were 65 males and 29 females, with ages ranging from 32 to 82 (median 64) years. Surgical procedures employed were total gastrectomy in 24 patients, proximal gastrectomy in 6, distal gastrectomy in 61, and partial gastrectomy in 3 patients. Size of main tumor at macroscopical examination of the resected stomach ranged from 10 to 200 (mean 28) mm.

Adjuvant chemotherapy was performed in 49 patients (preoperative in one, during surgery in 12, postoperative in 31, both during surgery and the postoperative period in 5) with a high risk for tumor recurrence, i.e., presence of lymph node metastasis, large tumor size, and tumor invasion to the serosa. Chemotherapeutic protocols were as follows: fluorouracil (5-FU) or its derivative alone in two patients; mitomycin C (MMC) alone in one; 5-FU and cisplatinum (CDDP) in nine; CDDP and MMC in six; 5-FU and MMC in three; 5-FU, MMC, and CDDP in four; 5-FU, MMC, and doxorubicin (DXR) in three; CDDP, MMC, and etoposide (VP-16) in seven; 5-FU, CDDP, and VP-16 in four; 5-FU, MMC, and epirubicin (EPI) in five; 5-FU, MMC, and cyclophosphamide (CTX) in two; 5-FU, CDDP, and CTX in two; 5-FU, MMC, VP-16, and CTX in one. Follow-up period for survivors ranged from 1 to 81 (median 30.1) months. The study was approved by the institutional review board of East Hospital, Tongji University.

Esopageal cancer. Sixty-four patients underwent surgery for ESCC at the Department of Surgery, Osaka University Hospital during the period from 2000 to 2005. There were 59 males and 5 females with ages ranging from 49-82 (median 65) years. Preoperative endoscopic examination of esophageal lesions was performed, and a histological diagnosis of ESCC was made based on the histological examination of biopsy specimens. Preoperative diagnostic examinations, including esophagography, computed tomography, and ultra-sound were performed for clinical staging of disease. All patients underwent curative surgery for ESCC: the resected esophagus was macroscopically examined to determine the location and size of the tumor. The size of the main tumor ranged from 1 to 390 mm (median 45 mm). None of patients received chemotherapy and radiation therapy before surgical resection.

Follow-up examinations. After surgery, all patients underwent laboratory examinations such as routine peripheral blood cell counts with measurement of the serum squamous cell carcinoma antigen levels in cases of ESCC every 1-6 months, and chest roentgenography, ultrasonography of the liver, computerized tomographic scan of the thorax and abdomen, and endoscopic examination of the remaining esophagus in cases of ESCC at intervals of 6-12 months.

Histological examination. Samples obtained from the gastric or esophageal lesions were fixed in 10% formalin and routinely processed for paraffin embedding. Histologic sections cut at 4 μm were stained with hematoxylin and eosin and immuno-peroxidase procedures. Histologic sections were analyzed by two investigators (Y.Q. and Y.T. for GC, and Y.Q. and E.M. for ESCC) to define the extent and mode of cancer invasion in the stomach or esophagus, lymph node metastasis, and histological subtype. Tumor stage was determined according to the pathologic TNM classification (25).

Immunohistochemistry. Avidin-biotin-peroxidase complex method (ABC) was used for immunostaining. Antigen retrieval was done with heating the sections in 10 mM citrate buffer for 10 min. Rabbit polyclonal anti-human PBX2 antibody (1:200, Santa Cruz, Santa Cruz, CA) was used as primary antibody. After washing with phosphate buffered saline (PBS), the sections were immersed in 2% hydrogen peroxidase in methanol for 20 min to block endogenous peroxidase activity, incubated with goat non-immune serum for 30 min, then incubated with primary antibody at room temperature for 1 h and at 4°C overnight. Sections were counterstained with hematoxylin. Immunostaining was carried out as described previously (24). Positive staining of endothelial cells in the same section was used as an internal positive control. For a negative control, primary antibody was replaced by non-immunized rabbit immunoglobulin G serum (Vector Laboratories, Burlingame, CA), giving uniformly negative results.

All immunohistochemically stained sections were examined in a blinded manner without any knowledge of the clinicopathologic parameters and patient outcome. Staining intensity of the tumor cells was compared to that of endothelial cells, and categorized as follows: equal to or stronger (level 1) and weaker (level 2) than that in the endothelial cells. Protein expression level was scored as the percentage of positive cells at 10 high power fields. Cases with level 1 expression in more than 20% of tumor cells were defined as 'high' and others as 'low' expression. Typical fields for high and low cases of GC and ESCC are shown in Fig. 1A.

Cell culture. Human gastric adenocarcinoma cell line (MNK-45) and human esophageal squamous cell carcinoma cell line (KYSE70) were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Nippon Bio-supp. Center, Tokyo, Japan; DMEM-10% FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

Table I. PBX2 expression and clinicopathological factors in 94 patients with GC.

Clinicopathological features	Category	No. of patients	PBX2 high expression (n=56)	PBX2 low expression (n=38)	P-value
Age, mean ± SD	Mean (64.10±13.09)		64.66±13.35	63.28±12.67	NS
Sex	Male	65	41	24	NS
	Female	29	15	14	
Tumor location	Upper third	23	19	4	NS
	Middle third	23	10	13	
	Lower third	48	27	21	
Tumor size, mm	<30	68	39	29	NS
	≥30	26	17	9	
Histological differentiation	Undifferentiated	55	35	20	NS
	Differentiated	39	21	18	
Vascular invasion	Absent	66	40	26	NS
	Present	28	16	12	
Lymphatic invasion	Absent	79	45	34	NS
	Present	15	11	4	
Depth of tumor invasion	pT1	15	7	8	NS
	pT2	17	8	9	
	pT3	30	17	13	
	pT4	32	24	8	
Lymph node metastasis	pN0	23	13	10	NS
	pN1	52	31	21	
	pN2	15	9	6	
	pN3	4	3	1	
Chemotherapy	Not performed	45	25	20	NS
	Performed	49	31	18	

GC, gastric cancer.

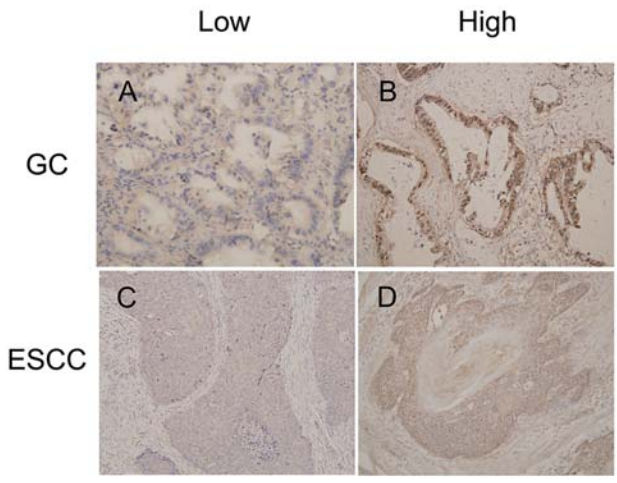

Figure 1. PBX2 expression in GC (A and B) and ESCC (C and D). Low expression in A and C, and high expression in B and D. ABC method, x200.

Construction of PBX2 siRNA plasmids and transfection. To generate a stably expressing si-RNA system, the BLOCK-iT Pol II miR RNAi expression vector kit (Invitrogen, Carlsbad, CA) was used. The plasmids producing siRNA were transfected into the MNK-45 and KYSE70 cells, respectively, with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Cells stably expressing the PBX2 si-RNA and control cells stably expressing vector alone were established in DMEM-10% FBS containing blasticidin (Invitrogen) at a concentration of 24 μg/ml in KYSE70 and MNK-45 cells. The knocked-down of PBX2 expression in the stable clones was determined by Western blot analysis.

Western blot analysis. Cells were washed with ice-cold PBS and lysed. Protein concentration was determined using a protein assay reagent (Bio-Rad, Hercules, CA). Equal amounts of proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride membranes (Millipore,

Table II. PBX2 expression and clinicopathological factors in 64 patients with ESCC.

Clinicopathological features	Category	No. of patient	PBX2 high expression (n=21)	pPBX2 low expression (n=43)	P-value
Age, mean±SD	Mean (66.08±7.02)		66.62±6.09	65.95±7.21	NS
Gender	Male	59	19	40	NS
	Female	5	2	3	
Tumor location	Cervix	2	2	0	NS
	Upper third of thorax	6	2	4	
	Middle third of thorax	32	7	25	
	Lower third of thorax	21	10	11	
	Abdomen	3	0	3	
Tumor size, mm	<40	25	6	19	NS
	≥40	39	15	24	
Histological differentiation	Well-differentiated	21	5	16	NS
	Moderately differentiated	31	10	21	
	Poorly differentiated	12	6	6	
Vascular invasion	Absent	39	14	25	NS
	Present	25	7	18	
Lymphatic invasion	Absent	20	6	14	NS
	Present	44	15	29	
Lymph node metastasis	Absent	31	7	24	NS
	Present	33	14	19	
Depth of tumor invasion	pT1	32	6	26	NS
	pT2	8	3	5	
	pT3	23	11	12	
	pT4	1	1	0	
Stage	I	24	5	19	NS
	IIA+B	19	5	14	
	III	14	6	8	
	IVA+B	7	5	2	

ESCC, esophageal squamous cell carcinoma.

Bedford, MA). Western blot analysis was carried out as described previously (26). Primary antibodies were used at dilution of 1:200 for anti-PBX2 and 1:1000 for anti-actin (Sigma). Density of each band was quantitated by Image J software.

WST-1 assay. To evaluate the effects of PBX2 on proliferation and apoptosis, cells ($1x10^4$) were cultured for 24 h with DMEM-10% FBS or with serum-free DMEM. Premix WST-1 cell assay system (Takara Bio Inc, Kyoto, Japan) was used for evaluation of proliferative activity and viability of cells cultured with and without FBS, respectively. The absorbance at 450 nm was measured at 0 and 20 min after WST-1 solution was added. The value at 20 min was divided with that at 0 min, and the resultant was designated as proliferation or viability index. Experiments were carried out in triplicate, and the results were shown as mean ± SD of three independent experiments.

Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) cell proliferation assay. The cells ($1x10^6$/ml) were washed and suspended in 100 μl PBS containing 0.1% BSA, and mixed with an equal volume of 50 μM CFDA-SE (Invitrogen, final concentration 25 μM). After incubation at 37°C for 20 min, 1 ml of DMEM medium with 10% FBS was added to stop the uptake of CFDA-SE dye. Labeled cells were cultured in DMEM-10% FBS, and the rate of decrease in fluorescence intensity, i.e. the proliferative activity, was analyzed on a FACS Calibur flow cytometer (BD, Franklin Lakes, NJ) for 4 days.

Table III. Univariate analysis of prognostic factors for DFS and OS in 94 pateints with GC.

Clinicopathological features	Category	No. of patients	5-year DFS rate (%)	P-value	5-year OS rate (%)	P-value
PBX2 expression	Low	38	70.7	<0.01	75.5	<0.01
	High	56	40.9		49.5	
Age	≤60	36	69.2	<0.01	77.0	<0.01
	>60	58	42.6		49.3	
Sex	Male	65	44.0	<0.05	51.3	<0.05
	Female	29	72.2		79.3	
Tumor location	Upper third	23	37.9	NS	42.1	<0.05[a]
	Middle third	23	56.5		60.5	<0.01[b]
	Lower third	48	58.1		68.4	
Tumor size, mm	<40	68	61.4	<0.005	66.9	<0.05
	≥40	26	30.7		41.9	
Histological differentiation	Undifferentiated	55	41.8	<0.01	50.7	<0.05
	Differentiated	39	68.7		73.3	
Vascular invasion	Absent	66	58.6	<0.05	65.8	<0.05
	Present	28	38.9		46.1	
Lymphatic invasion	Absent	79	59.1	<0.0001	65.1	<0.001
	Present	15	20.0		33.3	
Lymph node metastasis	pN_0	23	30.4	<0.001[c]	47.8	<0.01[d]
	pN_1	52	11.5	<0.0001[e]	26.9	<0.0001[f]
	pN_2	15	0.0		6.6	
	pN_3	4	0.0		0.0	
Depth of tumor invasion	pT1	15	93.3	<0.001[g]	100.0	<0.001[h]
	pT2	17	88.2		93.8	
	pT3	30	46.2		59.1	
	pT4	32	21.8		25.0	
Chemotherapy	Absent	45	48.6	NS	50.3	NS
	Present	49	56.6		68.9	

[a]1-2 vs. 3; [b]1 vs. 2-3; [c] and [d]1 vs. 2-4; [e] and [f]1-2 vs. 3-4; [g] and [h]1-2 vs. 3-4. DFS, desease-free survival; OS, overall survival; GC, gastric cancer; vs., versus.

Staining of apoptotic cells with annexin V. Apoptotic cells were stained with annexin V-FITC using the Apoptosis Detection kit (MBL, Nagoya, Japan), and subsequently analyzed by fluorescent microscope (Biozero, BZ-8000, Keyence, Japan).

Matrigel invasion assay. Invasion of tumor cells into the Matrigel was examined with BD BioCoat Matrigel invasion chamber (BD Biosciences, San Jose, CA). Briefly, cells were seeded in DMEM without FBS in the Matrigel invasion chamber, and cultured in DMEM-10% FBS for 60 h. Invading cells were stained with Diff-quick staining kit (Siemens, Munich, Germany). Number of invading cells was counted at five microscopic fields per well at a magnification of x100, and the extent of invasion was expressed as the average number of cells per mm^2.

In vitro colony formation assay. A total of $1x10^3$ cells were plated in 60 mm culture dishes and kept for 14 days in a medium with 15% FBS-containing methylcellulose, and the number of formed colonies was counted.

Real-time quantitative polymerase chain reaction (RT-QPCR). Total RNA was extracted from PBX2 knocked down cell lines of MNK-45 and KYSE70 using RNeasy RNA extraction kit (Qiagen, Valencia, CA) according to the manufacturer's protocol, and cDNA was synthesized using oligo (dT) primers and SuperScript III reverse transcriptase (Invitrogen). RT-QPCR was used to quantify mRNA expression of PBX2 and Bcl-2 using an ABI PRISM 7700 instrument (Applied Biosystems, Foster City, CA). GAPDH was used as a reference for gene amplification (Applied Biosystems).

Table IV. Multivariate analysis of prognostic factors for DFS and OS in 94 patients with GC.

Factor	Category	Relative risk	95% Confidence interval	χ^2 value	P-value
Overall survival					
PBX2 expression	Low High	1.683	1.122-2.625	6.414	<0.05
Age	≤60 >60	1.032	0.678-1.640	0.02	0.886
Sex	Male Female	1.094	0.695-1.858	0.135	0.713
Tumor location	Upper Middle+Lower	0.664	0.456-0.990	4.023	<0.05
Tumor size, mm	<40 ≥40	1.001	0.686-1.445	0.00001	0.995
Histological differentiation	Undifferentiated Differentiated	1.844	1.222-2.871	8.647	<0.005
Vascular invasion	Absent Present	1.149	0.797-1.640	0.573	0.449
Lymphatic invasion	Absent Present	1.108	0.729-1.627	0.25	0.617
Lymph node metastasis	pN_0+pN_1 pN_2+pN_3	2.129	1.406-3.221	12.381	<0.0005
Depth of tumor invasion	pT_3+pT_4 pT_1+pT_2	4.835	2.088-21.007	19.579	<0.0005
Disease-free survival					
PBX2 expression	Low High	1.84	1.274-2.761	11.091	<0.005
Age	≤60 >60	1.056	0.734-1.564	0.082	0.775
Sex	Male Female	1.04	0.701-1.62	0.034	0.854
Tumor size, mm	<40 ≥40	1.02	0.719-1.438	0.013	0.908
Histological differentiation	Undifferentiated Differentiated	1.673	1.174-2.450	8.261	<0.005
Vascular invasion	Absent Present	1.027	0.728-1.432	0.024	0.877
Lymphatic invasion	Absent Present	1.256	0.860-1.790	1.437	0.231
Lymph node metastasis	pN_0+pN_1 pN_2+pN_3	1.948	1.310-2.866	10.431	<0.005
Depth of tumor invasion	pT_3+pT_4 pT_1+pT_2	2.979	1.666-6.374	16.208	<0.0005

DFS, desease-free survival; OS, overall survival; GC, gastric cancer.

Table V. Univariate analysis of prognostic factors for DFS and OS in 64 patients with ESCC.

Factors	Category	No. of patients	5-year DFS rate (%)	P-value	5-year OS rate (%)	P-value
PBX2 expression	High	21	56.1	<0.01	56.4	<0.01
	Low	43	85.0		86.3	
Gender	Male	59	76.5	NS	77.6	NS
	Female	5	40.0		50.0	
Tumor location	Cervix	2	0.0	NS	0.0	NS
	Upper third of thorax	6	33.3		50.0	
	Middle third of thorax	32	37.5		37.5	
	Lower third of thorax	21	28.6		33.3	
	Abdomen	3	0.0		0.0	
Tumor size (mm)	<40	25	75.8	NS	78.2	NS
	≥40	39	74.8		73.9	
Histologic differentiation	Well-differentiated	21	33.3	<0.05[a]	38.1	<0.05[b]
	Moderately differentiated	31	32.3		35.5	
	Poorly differentiated	12	25.0		25.0	
Vascular invasion	Absent	39	86.9	<0.005	85.5	<0.05
	Present	25	55.4		59.7	
Lymphatic invasion	Absent	20	65.7	<0.05	69.5	NS
	Present	44	31.8		88.5	
Lymph node metastasis	Absent	31	86.4	<0.005	88.6	<0.005
	Present	33	42.9		36.4	
Depth of tumor invasion	pT1	32	43.8	<0.0001[c]	43.8	<0.01[d]
	pT2	8	25.0	<0.001[e]	37.5	<0.005[f]
	pT3	23	17.4		21.7	
	pT4	1	0.0		0.0	
Stage	I	24	45.8	<0.001[g]	45.8	NS
	IIA+B	19	21.1		21.1	
	III	14	14.3		14.3	
	IVA+B	7	42.8		57.1	

[a] and [b]1-2 vs. 3; [c] and [d]1 vs. 2-4; [e] and [f]1-2 vs. 3; [g]1-2 vs. 3-4. DFS, desease-free survival; OS,overall survival; ESCC, esophageal squamous cell carcinoma; vs., versus.

Mice and xenograft transplantation. Six to 8-week-old female non-obese diabetic (NOD)/Scid mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan) and kept under specific pathogen-free conditions. Before xenotransplantation, the mice were deeply anesthetized. All animal experiments were performed according to the guideline of Osaka University Animal Center, and approved by the institutional review board of committee of animal experiments (no. 753). For xenograft transplantation, KYSE70 cells ($1x10^4$) of PBX2 knocked down (KD1) and control (KC) were suspended in 0.2 ml of Matrigel (BD Biosciences), and were injected subcutaneously into NOD/Scid mice, respectively. The tumor volume was measured using the following formula: $(width)^2$ x (length)/2 according to the report by Meyer-Siegler *et al* (27).

Statistical analysis. Statistical analysis for experimental studies was carried out using Student's t-tests. The values are shown as the mean ± SE of at least three experiments. Statistical analyses for clinical samples were performed using JMP software (SAS Institute Inc., Cary, NC). Overall survival (OS) and disease-free survival (DFS) were measured from the date of surgery to death by any causes and to disease recurrence or occurrence of distant metastases, respectively.

Table VI. Multivariate analysis of prognostic factors for DFS and OS in 64 patients with ESCC.

Factors	Category	Relative risk	95% Confidence interval	χ^2 value	P-value
Overall survival					
PBX2 expression	High Low	1.966	1.087-3.703	5.005	<0.05
Histologic differentiation	Well+moderately Poorly	2.307	1.166-4.569	5.645	<0.05
Vascular invasion	Absent Present	2.146	1.210-3.985	6.776	<0.01
Depth of tumor invasion	pT1 pT2-4	1.664	0.875-3.669	2.347	0.126
Disease-free survival					
PBX2 expression	High Low	2.052	1.003-4.553	2.052	<0.05
Histologic differentiation	Well+moderately Poorly	0.920	0.481-1.783	0.065	0.800
Vascular invasion	Absent Present	1.981	1.008-1.149	3.930	<0.05
Lymphatic invasion	Absent Present	1.465	0.594-6.532	0.552	0.457
Depth of tumor invasion	pT1 pT2-4	2.680	0.984-12.149	3.710	0.054
Stage	I+II III+IV	0.890	0.463-1.811	0.117	0.732

DFS, desease-free survival; OS, overall survival; ESCC, esophageal squamous cell carcinoma; vs., versus.

Follow-up period for the survivors of GC and ESCC ranged from 1.8 to 94 (median 39.5) months and from 1.6 to 94 (median 46.4) months, respectively. The χ^2 and Fisher's exact tests were used to analyze the correlation between PBX2 expression determined by immunohistochemistry and clinicopathologic features of GC and ESCC. Kaplan-Meier method and log-rank tests were used to evaluate the survival rates and differences in survival curves. In the multivariate analysis, independent prognostic factors for survival of patients were determined by using Cox regression hazards model. $P<0.05$ were considered to be statistically significant.

Results

Clinicopathological features. The 94 cases of GC histologically consisted of 18 cases with well-differentiated, 19 moderately-differentiated, 48 poorly-differentiated adenocarcinoma, four signet ring cell carcinoma, three mucinous carcinoma, and one each of papillary carcinoma and medullary carcinoma. Cases with poorly-differentiated, signet ring cell, and mucinous carcinomas were unified and categorized as undifferentiated carcinoma. Other cases were unified as differentiated ones. Tumor cells invaded into mucosa or submucosa (pT_1) in 15 cases, muscularis propria or subserosa (pT_2) in 17, serosa (pT_3) in 30, and adjacent organs (pT_4) in 32. Seventy-two cases were node-negative and 23 node positive. In GC samples, 56 (59.6%) and 38 (40.4%) cases showed high and low PBX2 expression, respectively (Fig. 1A and B). None of the clinicopathological features correlated with PBX2 expression level (Table I).

In the 64 cases of ESCC, 21 had well differentiated, 31 moderately differentiated, and 12 poorly differentiated squamous cell carcinomas. Tumor cells invaded into the mucosa or submucosa (pT_1) in 32 cases, muscularis propria or subadventitia (pT_2) in 8, adventitia (pT_3) in 23, and adjacent organs (pT_4) in one. Twenty-one (32.8%) and 43 (67.2%) cases showed a low and high PBX2 expression, respectively (Fig. 1C and D). None of the clinicopathological features correlated with PBX2 expression level (Table II).

Univariate and multivariate analysis for prognostic factors of GC or ESCC. Five-year DFS and OS rate of the GC patients was 53.1 and 60.6%, respectively. Tumor recurrence was observed in 44 patients; peritoneum in eight, lymph nodes in nine, liver in six, lung in four, colon in four, esophagus in two, liver in three, bone in one, and multiple visceral meta-

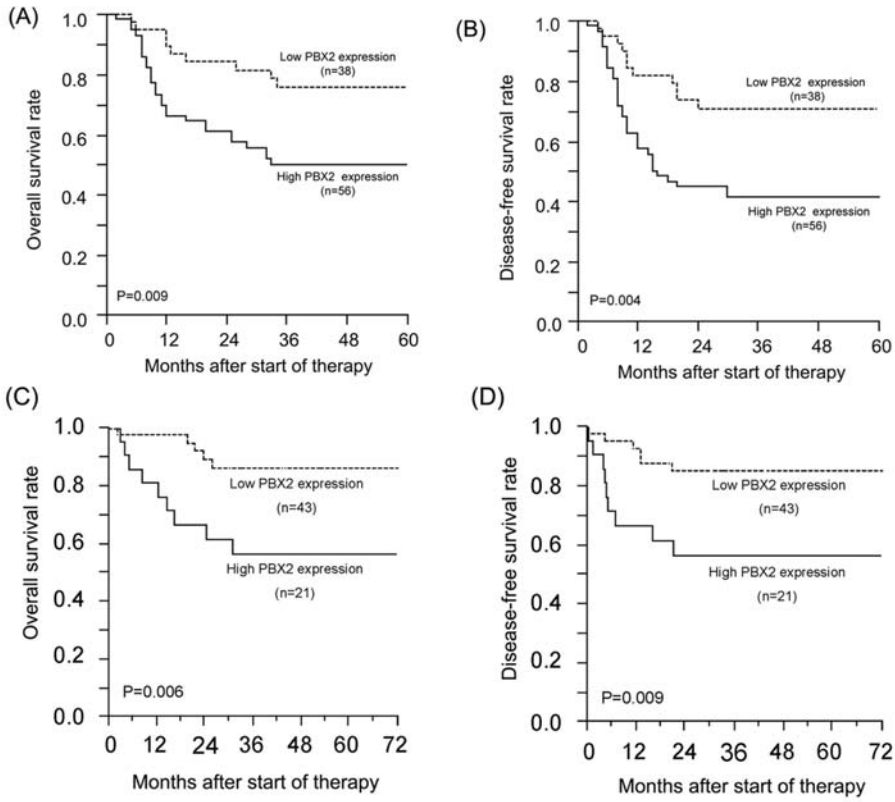

Figure 2. Kaplan-Meier plots of OS and DFS. Kaplan-Meier plots of OS and DFS of patients with high and low expression in GC (A and B) and ESCC (C and D).

stases in seven. Twenty-two patients died due to tumor. The univariate analysis revealed that gender and age of patients, size of tumor, tumor location, histologic differentiation, tumor invasion beyond propria muscle, vascular and lymphatic invasion, lymph node metastasis, and PBX2 expression were significant factors for both DFS and OS (Table III). Patients with high PBX2 expression showed worse OS and DSF than those with low expression (Fig. 2A and B). Multivariate analysis with factors proven to be significant in the univariate analysis revealed that PBX2 expression, depth of tumor invasion, histological differentiation, and lymph node metastasis were independent prognostic factors for both DFS and OS. Tumor location is an independent prognosticator for OS but not for DFS (Table IV).

As for ESCC patients, the 5-year DFS and OS rate was 57.1 and 61.1%, respectively. ESCC patients with low PBX2 expression had better OS and DFS than those with high expression (Fig. 2C and D). Univariate analysis revealed that histologic differentiation, depth of tumor invasion, vascular invasion, and lymph node metastasis were significant factors for both DFS and OS. Lymphatic invasion and stage significantly affected DFS but not OS (Table V). Multivariate analysis with factors proven to be significant in the univariate analysis revealed that PBX2 expression level in tumor cells and vascular invasion were independent prognostic factors for both DFS and OS, and histologic differentiation for OS (Table VI).

Knocked-down expression of PBX2 in GC and ESCC cell lines. To examine the effects of PBX2 on GC and ESCC cells, expression of PBX2 was knocked-down in GC cell line MNK-45 and ESCC cell line KYSE70. Four stable clones of independent PBX2 knocked-down cells were obtained from MNK-45 (MD1 and MD2) and KYSE70 (KD1 and KD2), respectively. When compared to control clones from the MNK-45 (MC) and KYSE70 (KC), expression amount of PBX2 protein in the knocked-down clones reduced (Fig. 3A and B). Effect of PBX2 on the cell proliferation was subsequently examined with WST-1 assay. No remarkable changes of proliferative activity were found between the control and knocked-down cells (Fig. 4A). For further characterization of cell proliferation, the control and knocked-down cells were labeled with CFDA-SE fluorescent dye, and time-dependent diminishment of fluorescent intensity was analyzed. When a single cell is divided into two, the original fluorescent intensity

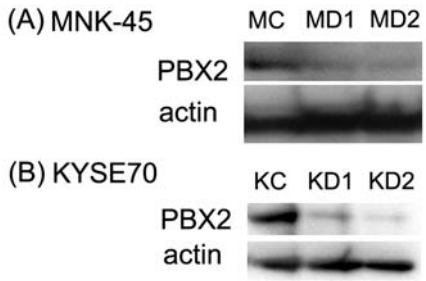

Figure 3. Expression of PBX2 in MNK-45 and KYSE70 cells examined by WB. (A) Amount of PBX2 protein in the control (MC) and si-RNA knocked-down (MD1 and MD2) MNK-45 cells. (B) Amount of PBX2 protein in the control (KC) and si-RNA knocked-down (KD1 and KD2) KYSE70 cells.

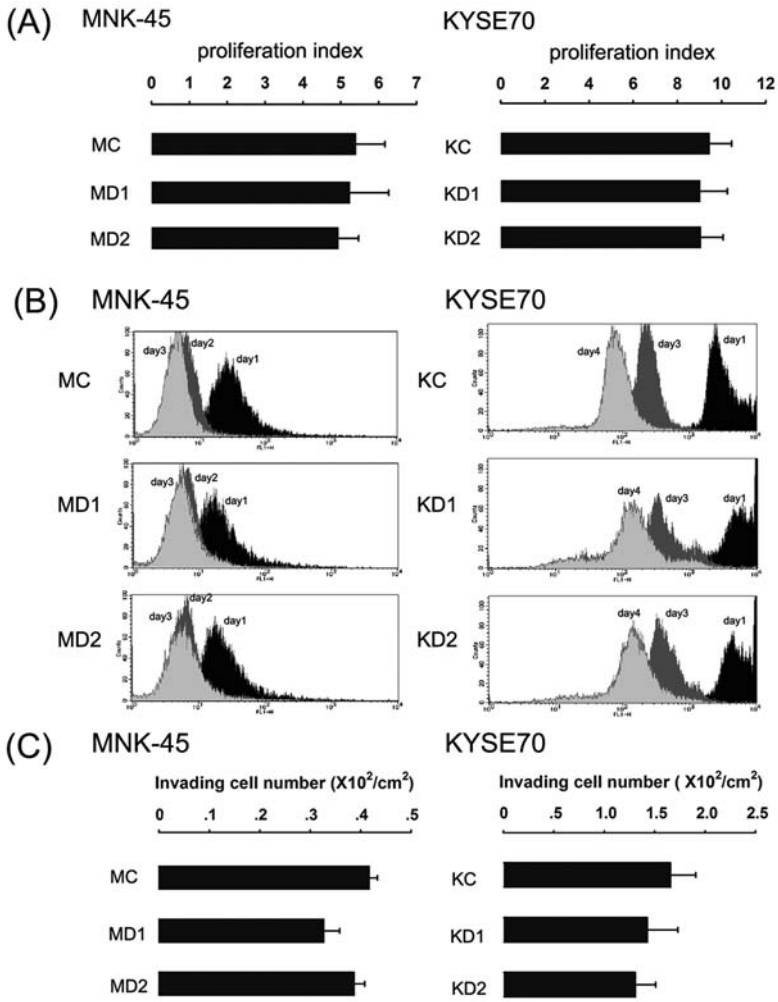

Figure 4. Effect of PBX2 expression on the proliferative and invasion activity in MNK-45 and KYSE70 cells. (A) Comparison of proliferation of PBX2 knocked down cells to that of control cells. (B) Cell proliferative activity as revealed by CFDA-SE in MNK-45 and KYSE70 cells for 3 days. (C) Comparison of invasion activity of PBX2 knocked down cells to that of control cells.

decreases to one half, showing that the proliferative ability correlated with the diminishing rate of fluorescent intensity. No obvious differences in diminishing rate were detected between the control and knocked-down cells (Fig. 4B). These findings indicate that PBX2 did not show any effect on proliferation rate of GC and ESCC cell lines. Next, the effect of PBX2 on invasion ability was examined with Matrigel invasion assay: number of invading cells in knocked-down cells reduced, but the difference between the control and knocked-down cells was not significant (Fig. 4C).

Effect of PBX2 on colony formation and tumorigenic abilities. The effects of PBX2 on *in vitro* colony formation and *in vivo* tumorigenic activities were evaluated. As compared to the control cells, the PBX2 knocked-down cells formed lower number of colonies in both GC and ESCC cell lines (Fig. 5A). Next, $1x10^6$ KC and KD1 cells were injected into the left and right flank of 2 NOD/Scid mice. After 5 weeks, tumors were resected. The sizes of tumors derived from PBX2 knocked-down cells were significantly smaller than those of the control cells (Fig. 5B). Histologically, both tumors derived from the control and knocked-down cells showed squamoid pattern of proliferation (Fig. 5C).

Vulnerability to apoptosis in PBX2-knocked down cells. Resistance to apoptotic stimuli was analyzed in PBX2 knocked-down cells. Under serum-depleted condition, viability of PBX2 knocked-down cells decreased more than that of control cells (Fig. 6A). Content of early apoptotic cells was examined with annexin V staining. Amount of early apoptotic cells was significantly higher in PBX2 knocked-down cells than in control cells of GC and ESCC (Fig. 6B and C).

To examine why the content of apoptotic cells increased by PBX2 knocked-down, expression level of Bcl-2, which plays an important role for elongation of programmed cell death, was examined in the GC and ESCC cell lines. The real-time RT-PCR revealed that the amount of Bcl-2 mRNA in PBX2 knocked-down cells was significantly decreased compared to the control cells in both GC and ESCC cell lines (Fig. 7A and B).

Discussion

The patient characteristics, such as gender, age, stage, histologic type, and 5-year survival rate in the present series were similar to those in the previous reports from China, Japan, and other countries where GC and ESCC are common

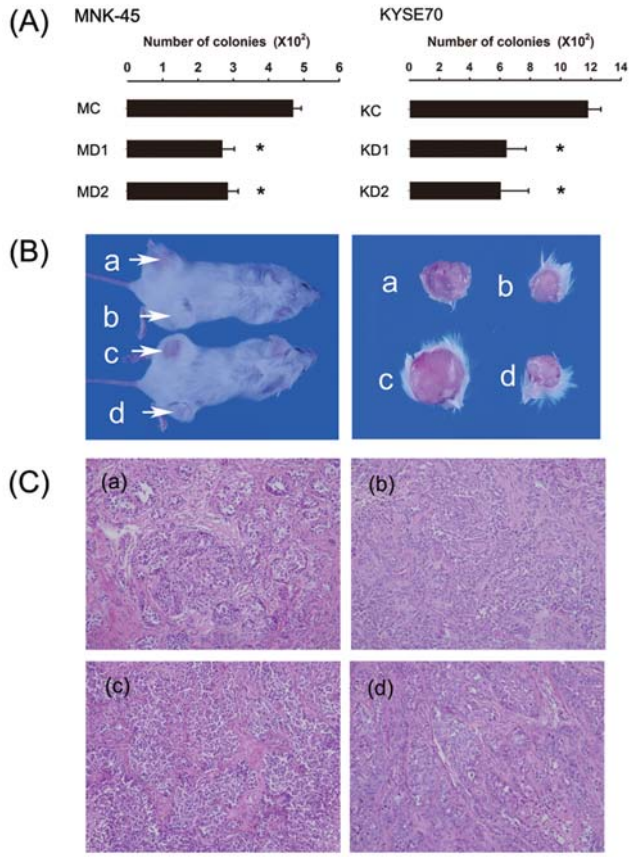

Figure 5. Effects of PBX2 on *in vitro* colony formation and *in vivo* tumorigenic activities. (A) Number of colonies was compare between the control and the PBX2 knocked-down cells (B) Tumors in NOD/Scid mice at the injection sites of the control (a and c) and the knocked-down (b and d) KYSE70 cells. (C) Histology of tumor derived from the control (a and c) and the knocked-down (b and d) cells. x400 The values in (A) represent the mean ± SE of three experiments. *p<0.01 by the Student's t-test.

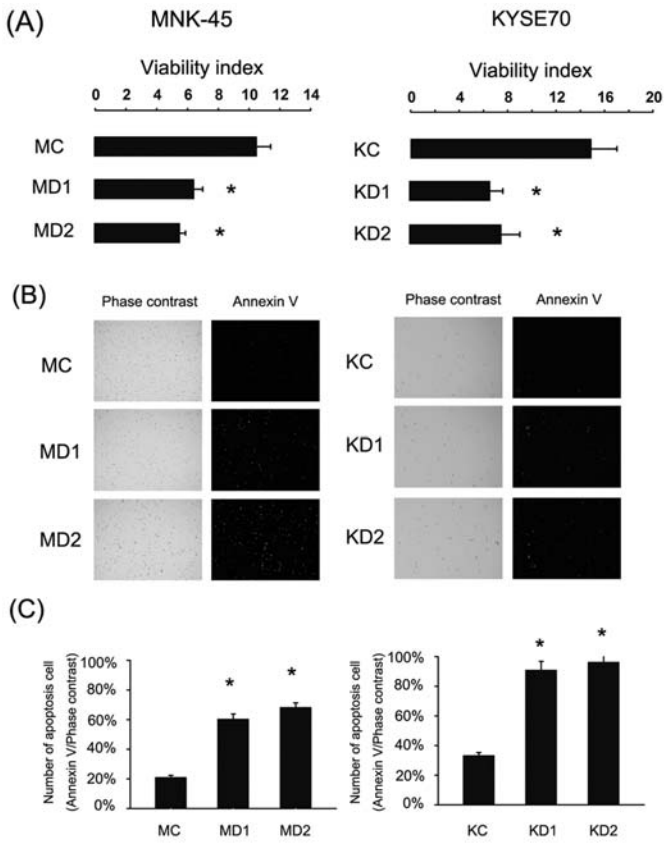

Figure 6. Effects of PBX2 expression on apoptosis of MNK-45 and KYSE70 cells. (A) Comparison of viability of PBX2 knocked-down cells to that of control cells. (B) Cells in apoptosis were compared between PBX2 knock-down and control cells. (C) Comparison of apoptotic cell proportion of PBX2 knocked-down cells to that of control cells. The values in (C) represent the mean ± SE of three experiments. *p<0.001.

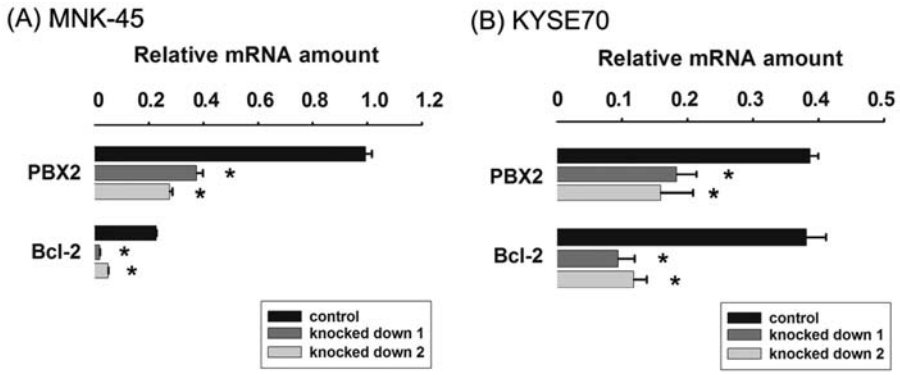

Figure 7. Expression of Bcl-2 gene in PBX2 knocked-down MNK-45 (A) and KYSE70 (B) cells. Relative amounts of PBX2 and Bcl-2 mRNA were shown. Amounts of Bcl-2 mRNA significantly reduced when PBX2 expression levels decreased. *P<0.05 by Student's t-test.

(28-30). In addition, the present study also showed that clinicopathological variables, such as tumor invasion, occurrence of metastasis, vascular and lymphatic invasion, and serosal invasion were significant factors for prediction of long-term survival of GC or ESCC patients as reported previously (28,31,32). These findings indicate that the results obtained from the present cases are commonly applicable. Our previous study showed that the high expression of PBX2 in the tumor cells was an indicator of poor prognosis in NSCLC (24). The present study also showed the prognostic significance of PBX2 expression level for both GC and ESCC, but the effect of PBX2 expression on survival was more profound, PBX2 expression was an independent prognostic indicator for GC and ESCC.

PBX2 protein, a main member of PBX family, is ubiquitously expressed in various tissues with different expression

levels by tissues. PBX2 works as a cofactor with other proteins, such as HOX, and forms dimeric complexes with increasing DNA binding affinity and specificity (33). HOX genes are essential for all aspects of mammalian growth and differentiation. Several studies indicated that PBX proteins function *in vivo* as regulators of differentiation and function in concert with HOX proteins in development (34). These protein complexes, including PBX2 protein, regulate the expression of number of genes, thus induce the execution of various cellular functions, such as anti-apoptosis, and inhibition of cell differentiation and proliferation (35).

It has been shown that deregulated expression of HOX genes is involved in carcinogenesis, i.e., the high expression of HOX correlated with poor prognosis of patients in acute myeloid leukemia and lymphoid malignancies (13,36,37). Several studies showed significantly higher level of HOX expression in cancer cells compared to normal mucosa in ESCC and GC (6,38). The synergistic effects of HOX proteins and PBX2 protein findings explain why PBX2 expression correlates with poor prognosis of GC and ESCC patients. PBX2 could play a significant role in tumor development.

To evaluate functional sequels induced by PBX2 expression, PBX2 siRNA plamids were constructed and transfected into the GC and ESCC cell lines. The knocked-down expression of PBX2 resulted in decrease of *in vitro* colony formation and *in vivo* tumorigenic activities. Correlation of PBX2 expression level with tumorigenic activity of the cultured cells was consistent with findings found in clinical cases that tumor recurrence rate was higher in the cases with high PBX2 expression than those with low expression. PBX2 expression level was not correlated with tumor size, frequencies of lymph node metastasis and vascular and lymphatic invasions in patients of GC and ESCC. These findings suggest that PBX2 might not promote tumor proliferation and lympho-vascular invasion.

The knocked-down expression of PBX2 did not change proliferation and invasion capacities of the cultured cells, but increase of apoptosis rate and the decrease of Bcl-2 expression were found. These findings suggested that PBX2 expression accelerated tumor growth through escape from apoptosis. Supporting evidence for this has been reported. Potts *et al* reported that HOX protein ensures cell survival by inhibition of apoptosis, thus promotes leukemia development (39). Decreased activity of HOX proteins by interfering their binding to the PBX co-factor causes apoptosis of cancer cell lines *in vitro* and reduces the growth *in vivo* (15,40).

In conclusion, the present study clearly demonstrated that the increased expression of PBX2 in GC and ESCC is a sign for poor prognosis and PBX2 is an independent prognosticator for GC and ESCC patients. PBX2 might promote tumor growth through escape of cancer cells from apoptosis.

Acknowledgements

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