Prognostic significance of pre B cell leukemia transcription factor 2 (PBX2) expression in non-small cell lung carcinoma

Ying Qiu,[1,2] Eiichi Morii,[2,7] Yasuhiko Tomita,[3] Binglin Zhang,[2] Akihide Matsumura,[4] Masanori Kitaichi,[5] Meinoshin Okumura[6] and Katsuyuki Aozasa[2]

[1]Department of Pathology, Tongji University School of Medicine, Siping Road, Shanghai 200092, China, Departments of [2]Pathology and [6]Respiratory Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; [3]Department of Pathology, Osaka Medical Center for Cancer and Cardiovascular Diseases, 1-3-3, Nakamichi, Higashinari, Osaka, Osaka 537-8511, Japan; Departments of [4]Surgery and [5]Pathology, Kinki-Chuo Chest Medical Center, 1180 Nagasonecho, Kita, Sakai, Osaka, 591-8555, Japan

(Received November 11, 2008/Revised March 2, 2009/Accepted March 4, 2009/Online publication April 1, 2009)

Previous studies on the mammary carcinoma cell line have shown that the pre B cell leukemia transcription factor 1 (PBX1) was a transcription factor for valosin-containing protein (VCP), which is involved in invasion and metastasis of cancers. The roles of PBX1 and PBX2, a highly homologous transcription factor to PBX1, for expression of VCP were examined in the cell lines from non-small cell lung cancer (NSCLC). The effects of PBX1 and PBX2 on VCP expression were examined with siRNA in A549 and PC14 NSCLC cell lines. Expression levels of PBX2 and VCP were immunohistochemically examined and compared with each other in 206 NSCLC cases. Subsequently, significance of PBX expression in clinical behavior of NSCLC patients was evaluated. Expression levels of VCP mRNA significantly decreased when PBX2 but not PBX1 expression was knocked down in NSCLC cell lines. Immunohistochemically, staining intensity of PBX2 was correlated with that of VCP in clinical samples. Then correlation of PBX2 expression and clinical behavior of NSCLC patients was evaluated. Univariate analysis revealed high expression levels of PBX2 and VCP to be poor prognosticators for overall and disease-free survival. Multivariate analysis revealed that high expression of VCP but not PBX2 to be an independent prognostic factor. PBX2 is a transcription factor for VCP in NSCLC. Because high levels of PBX2 expression correlated with prognosis of NSCLC, PBX2 could be a target molecule for treatment of NSCLC. (*Cancer Sci* 2009; 100: 1198–1209)

Lung cancer has become the most common cause of cancer death in the world.[1] Non-small cell lung cancer (NSCLC) comprises 80% of all lung cancers.[2] Despite the advances in the methods of detection and treatment for lung cancer, prognosis of patients still remains unfavorable.[3]

Correlation between expression of valosin-containing protein (VCP) in tumors and patients' prognosis regarding NSCLC has been reported previously.[4] VCP, initially identified as a gene related to metastasis,[5] is known to be involved in the ubiquitin/proteasome-degradation pathway, which works in both upregulation of cell proliferation and anti-apoptosis.[6,7] VCP expression level correlated with the recurrence rate and prognosis of patients with several cancers other than NSCLC such as hepatocellular,[8] gastric,[9] colon,[10] and gingival[11] cancer. Pre B cell leukemia transcription factor 1 (PBX1) works as a transcription activator for VCP expression in the breast cancer cell line, thus the PBX1-VCP pathway may be important for cell survival at least in breast cancer.[12]

PBX1 was first identified as the chromosome participant of (1;19) translocation,[13] found in 20% of pediatric pre B acute lymphoblastic leukemia.[13,14] This translocation-created chimeric gene induces tumorigenic conversion in NIH3T3 fibroblasts.[15,16] PBX2 was identified as a highly related homolog to PBX1.[17] PBX1 and PBX2, which are important members of the PBX family, have recently been shown to function as a transcription factor in cooperation with Homeobox (Hox) proteins in the regulation of proliferation and differentiation of normal and cancer cells.[18,19] Especially, PBX2 was reported to be expressed strongly in the nucleus: its nuclear localization is regulated through the interaction with other factors such as Pbx-regulating protein (PREP-1) or Myeloid ecotropic viral integration site 1 homologue (MEIS1). It could be postulated that the PBX2, as a transcription factor, may play a role for up-regulation of tumor-associated protein expression.

In the present study, the role of PBX for VCP expression was examined with siRNA in NSCLC cell lines, and with immunohistochemistry on clinical samples from NSCLC. The clinical relevance of PBX expression in NSCLC was evaluated.

Materials and Methods

Cell culture. NSCLC cell lines, A549 and PC-14 (obtained from Human Science Research Resources Bank, Osaka, Japan) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2.

Transient transfection. On the day before the transfection, the A549 and PC14 cells were seeded onto 6-well plates at a density of 2×10^5 cells per well, respectively. The cells were transfected with 5 μM small interfering RNA (siRNA) target sequences of PBX1 and PBX2 using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, US) according to the manufacturer's instructions. siRNA target sequence to PBX1 and PBX2 (Applied Biosystems, Foster City, CA, US) was 5′-GAAGAACAUAGGUAAAUUU-3′ and 5′-GAGGAUUCGCUAUAAGAAA-3′, respectively. As a control, negative control siRNA (Applied Biosystems) was used.

Real-time quantitative polymerase chain reaction (RT-QPCR). After 48 h of transient transfection, total RNA was extracted from NSCLC cells using RNeasy RNA extraction kit (Qiagen,

[7]To whom correspondence should be addressed.
E-mail: morii@patho.med.osaka-u.ac.jp

Abbreviations: PBX, pre B cell leukemia transcription factor; VCP, valosin-containing protein; NSCLC, non-small cell lung cancer; Hox, homeobox; siRNA, small interfering RNA; ChIP, chromatin immunoprecipitation assay; RT-QPCR, real-time quantitative polymerase chain reaction; nt, nucleotide; WB, Western blot; WHO, World Health Organization classification; TNM, tumor-node-metastasis; IHC, immunohistochemistry; ABC, avidin-biotin-peroxidase complex; PBS, phosphate buffered saline; OS, overall survival; DFS, disease-free survival.

doi: 10.1111/j.1349-7006.2009.01156.x

Valencia, CA, US) 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 PBX1, PBX2, and VCP using an ABI PRISM 7700 instrument (Applied Biosystems). RT-PCR was carried out using Taqman probe/primer sets specific for human PBX1, PBX2, and VCP. GAPDH was used as a reference for gene amplification (Applied Biosystems).

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was performed using a kit purchased from Upstate (Lake Placid, NY, US) according to the manufacturer's protocol. A549 cells were treated with 1% formaldehyde for 10 min. The chromatin was subjected to sonication for shearing chromatin to 200–1000 bp fragments. Soluble chromatin was incubated with either anti-PBX2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, US) or preimmune IgG serum. DNA was purified with washing and elution, then 2 μL of DNA solution was used as a template for 35 cycles of PCR amplification with primers to amplify VCP promoter ranging between nt-27 and -237: 5′-CAAACGACGGTCGCAGACGCT-3′ and 5′-TTACTAGGCGT-GTCGCATCAC-3′. PCR products were sequenced to confirm the accuracy of the assay.

Plasmids. The DNA fragment encoding the entire open reading frame of PBX2 was obtained by PCR from the reverse-transcribed product of A549 cells. The used primers were 5′-ATGGACGA-ACGGCTACTGGGGCCGCCCCC-3′ and 5′-TCAGTTGGAGGT-ATCAGAGTGAACACTCC-3′. The amplified DNA fragment was subcloned into pIRESpuro3 (Takara Bio, Kyoto, Japan). All constructs were sequenced to confirm the accuracy of mutagenesis.

Transfection and luciferase assays. The reporter plasmids that contained the promoter region of the VCP gene starting from nucleotide (nt)-1157 with normal or mutated PBX binding sites, and the reporter plasmid with the minimal VCP promoter starting from nt-107 were described previously.[12] The PBX2 expression plasmid was used as an effector and the back-born pRIESpuro3 vector was used as a negative control. The luciferase reporter (0.5 μg) and effector plasmids (0.25 μg) were co-transfected into NSCLC cells using Lipofectamine 2000. The relative luciferase activity was examined with the dual luciferase reporter assay system (Promega) according to the manufacturer's protocol. The normalized value was divided by the value obtained without effector plasmids, which was expressed as the relative luciferase activity.

Western blot (WB) analysis. Nuclear protein of resected tumors from seven patients with NSCLC was used. WB analysis was carried out as described previously.[12] Primary antibodies were used at dilution of 1×200 for anti-human PBX2 and 1×500 for acetyl-Histon H3 (Upstate). Histon H3 was used for quantifying the amount of loaded protein in nuclear fractions. The intensity of each band was quantified with NIHimage (http://rsbweb.nih.gov/nih-image/), and the ratio of band intensity of PBX2 to Histon-H3 was shown as the relative amount of PBX2 protein.

Patients and tissue samples. Two hundred and six patients who underwent surgery for NSCLC at the Division of Surgery, National Kinki Chuo Hospital during the period from April 1990 to December 1995 were examined. There were 132 males and 74 females with ages ranging from 35 to 78 years (median, 62). Surgeries employed were left lower lobectomy in 22 patients, left upper lobectomy in 41, left pneumonectomy in 7, left lower segmentectomy in 1, right upper lobectomy in 76, right lower lobectomy in 28, right middle lobectomy in 16, right middle and lower lobectomy in 7, and right pneumonectomy in 8. Resected specimens were macroscopically examined to determine the location and size of the tumors. The size of tumor ranged from 7 to 110 mm (median, 31.3). Samples obtained from the lung lesions and dissected lymph nodes were fixed in 10% formalin and routinely processed for paraffin embedding. Histologic sections cut at 4 μm were stained with hematoxylin and eosin and immunoperoxidase procedures. Extent and mode of cancer invasion, lymph node metastasis, and histologic subtype of NSCLC were defined based on the World Health Organization classification (WHO).[20] Stage of the disease was determined according to tumor-node-metastasis (TNM) classification.[21]

Adjuvant therapy was performed in 33 patients at high risk of tumor recurrence, that is, occurrence of lymph node metastasis, huge tumor (diameter >5 cm), and tumor invasion to the visceral pleura. The protocols were as follows: preoperative chemotherapy with *cis*-platinum, vindesine, and mitomycin C in 2 patients; postoperative chemotherapy with *cis*-platinum and vindesine in 5; 5-fluorouracil derivative (uracil 1,2-tetrahydrofuranyl-fluorouracil) alone in 18; postoperative immunotherapy with bestatin in 7 and OK-432 in 1. All patients were followed up with laboratory examinations including routine peripheral blood cell counts at 1- to 6-month intervals, chest roentgenogram, computed tomographic scan of the chest, and endoscopic examinations of the bronchus at 6–12 month intervals. Follow-up period for survivors ranged from 45 to 150 months (median, 100). This study was approved by the ethical review board of Kinki-Chuo Chest Medical Center.

Immunohistochemistry (IHC) and evaluation of staining score. IHC was carried out as previously described.[4] A standard avidin-biotin-peroxidase complex (ABC) method was used for immunostaining. Histologic sections were deparaffined and treated with microwave oven heating at a high power for 10 min in a 10-mM citrate buffer to retrieve antigenicity. After washing with phosphate buffered saline (PBS), the sections were immersed in 2% hydrogen peroxidase in methanol for 20 min to block any endogenous peroxidase activity, followed by 30 min of incubation with goat non-immune serum. Sections were incubated with primary antibody for 1 h at room temperature and overnight at 4°C. Primary antibodies used were anti-human PBX2 antibody (1:200) and anti-human VCP antibody (1:100) (Santa Cruz Biotechnologies). Positive staining in endothelial cells was used as an internal positive control. For negative controls, non-immunized mouse serum (Vector Laboratories, Burlingame, CA, US) was used as the primary antibody, giving uniformly negative results.

To evaluate the specificity of primary antibodies, the pre-absorption of anti-PBX2 and anti-VCP antibodies with the peptide of PBX2 and VCP (Santa Cruz Biotechnologies; 1:5), respectively, were performed. Then, IHC was done using the pre-absorbed antibodies, and the resultant signals were compared to those of non-absorbed antibodies.

Stained sections were evaluated independently by two investigators (Y.Q. and Y.T.). Endothelial cells showed a positive staining with a constant intensity; the staining intensity of tumor cells was categorized as equal to or stronger (level 2) and weaker (level 1) than that in endothelial cells. VCP expression was detected only in cytoplasm, whereas PBX2 expression was observed both in nucleus and cytoplasm. Since the role of PBX2 as a transcription factor was examined in the present study, the nuclear staining was evaluated. Protein expression level was graded as the percentage of positive cells at 10 high-power fields. Cases with level 2 expression in more than 20% of tumor cells were defined as 'high' and others as 'low'.

Statistical analysis. Data of RT-PCR and luciferase assays were expressed as mean ± SE. The significance of differences of the mean values was determined by Student's *t*-test. All experiments were performed in triplicate. Statistical analyses for clinical samples were performed using JMP software (SAS Institute Inc., Cary, NC, US). The Chi-square and Fisher's exact probability test were used to analyze the correlation between PBX expression and clinicopathologic features of NSCLC. Kaplan-Meier

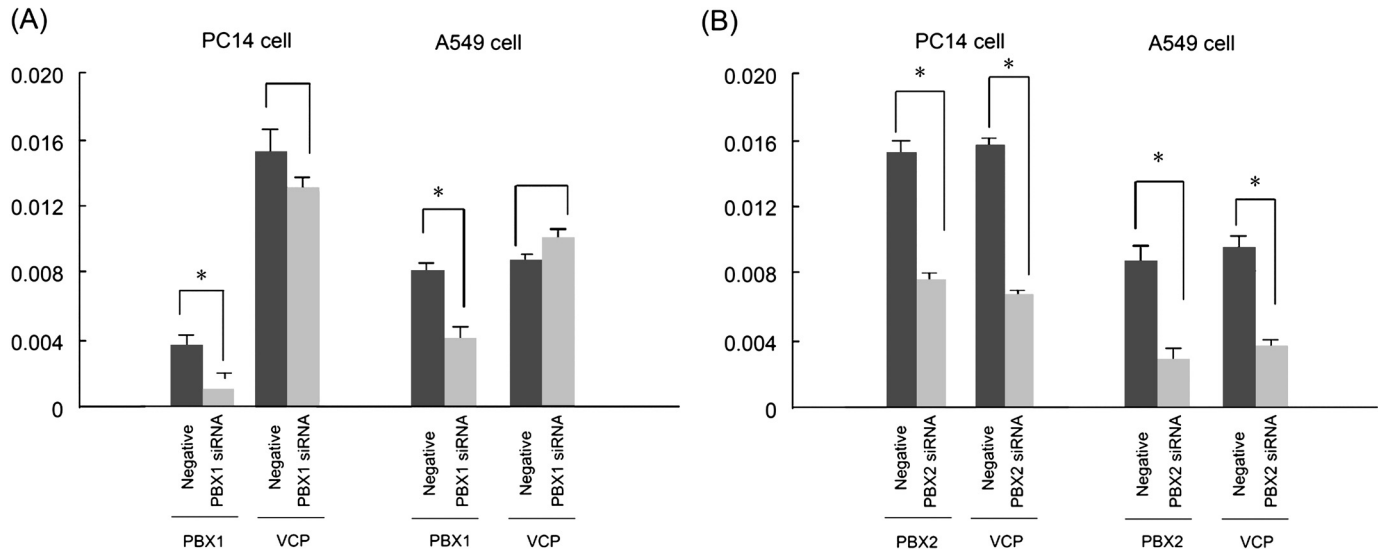

Fig. 1. Effect of pre B cell leukemia transcription factor 1 (PBX1) and PBX2 on valosin-containing protein (VCP) expression evaluated by siRNA and RT-PCR analysis on non-small cell lung cancer (NSCLC) cell lines PC14 and A549. (A) Relative amounts of PBX1 and VCP mRNA to GAPDH mRNA were shown. Decrease in PBX1 expression level by siRNA did not affect the amount of VCP mRNA. (B) Relative amounts of PBX2 and VCP mRNA were shown. The amount of VCP mRNA significantly reduced when PBX2 expression level decreased. *$P < 0.05$ by Student's test.

methods with the log-rank test were used to calculate overall survival (OS) and disease-free survival (DFS) rate, and to compare the differences in survival curves. Cox's proportional hazards regression model with a stepwise manner was used to analyze the independent prognostic factors. *P*-values of less than 0.05 were considered to be statistically significant.

Results

Changes of VCP expression by knocked-down PBX1 and PBX2. To test whether PBX1 and PBX2 regulates VCP transcription, the expression of each transcription factor was knocked down using siRNA in PC14 and A549 NSCLC cell lines. Decrease in PBX1 expression level induced by the siRNA did not affect the amount of VCP mRNA (Fig. 1A). In contrast, the amount of VCP mRNA reduced when PBX2 expression level decreased (Fig. 1B). These findings indicate that the expression level of PBX2 but not PBX1 was correlated with that of VCP, suggesting that PBX2 regulates VCP expression in the NSCLC cell lines. Then, we focused the study on the relation of PBX2 with VCP expression and its clinicopathological relevance in NSCLC.

Binding of PBX2 to the 5′-flanking sequence of VCP gene. ChIP assay was performed to examine whether PBX2 bound to the 5′-flanking sequence of VCP gene in A549 NSCLC cell line. After treatment with formaldehyde to crosslink protein and DNA, soluble chromatin extracted from A549 cells was immunoprecipitated with anti-PBX2 antibody. When DNA extracted from the immunoprecipitated product was used as a template, the PBX2 binding motif was successfully amplified by PCR (Fig. 2). The amplified fragment was confirmed to be the VCP promoter region by sequencing. No bands were detected when PCR was performed using DNA precipitated with pre-immune serum. These results showed the binding of PBX2 to the 5′-flanking sequence of VCP gene.

Increase of reporter activity of VCP promoter by PBX2 overexpression. Four PBX binding motifs were present in the 5′-flanking region of VCP gene. To examine whether PBX2 transactivated VCP promoter, the following three reporter plasmids

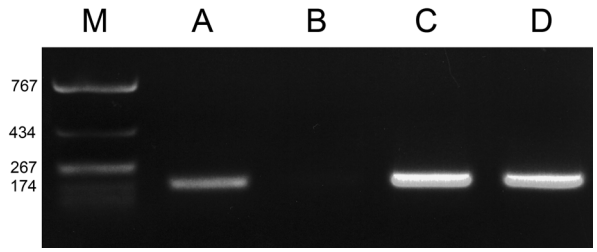

Fig. 2. Analysis of pre B cell leukemia transcription factor 2 (PBX2) binding to the 5′-flanking region of the human valosin-containing protein (VCP) gene. ChIP assay was performed using non-small cell lung cancer (NSCLC) cells. Input DNA sample with (lane A) or without (lane B) the addition of anti-PBX2 antibody was immunoprecipitated with anti-PBX2 (lane C) or preimmune IgG serum (lane D). Precipitated DNA fragments were PCR-amplified with primers specific for the VCP promoter region. PCR products were separated on 2% agarose gel and stained with ethidium bromide. Positive band in the lane of the sample precipitated with anti-PBX2 antibody (lane A) was seen at the same size with those of input DNA (lanes C and D). M; molecular weight marker.

were used; the plasmid with intact four PBX binding motifs, the plasmid mutated at four motifs, and the plasmid deleting these (Fig. 3). These reporter plasmids were transfected to A549 and PC-14 NSCLC cell lines, expressing PBX2 and VCP. When the PBX binding motifs were mutated or deleted, the reporter activity decreased in A549 cells, indicating that the PBX2 transactivated the promoter activity of VCP gene through PBX binding motifs (Fig. 3A). The reporter activity of plasmid containing PBX binding motifs significantly increased when PBX2 expression plasmid was co-transfected (Fig. 3A). When PBX binding motifs were mutated or deleted, the increase of reporter activity by PBX2 overexpression was abolished (Fig. 3A). These findings indicate that the PBX2 transactivated VCP promoter through its binding motifs. Comparable results were obtained in PC14 cells (Fig. 3B).

doi: 10.1111/j.1349-7006.2009.01156.x

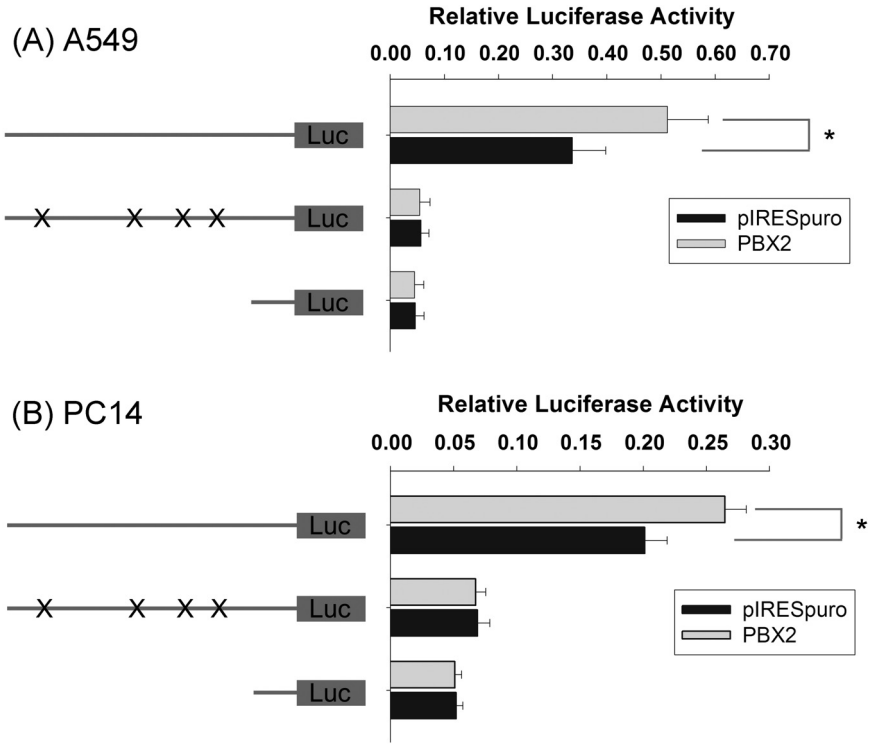

Fig. 3. Pre B cell leukemia transcription factor 2 (PBX2) overexpression up-regulated luciferase activity of valosin-containing protein (VCP) promoter in non-small cell lung cancer (NSCLC) cells of A549 (A) and PC14 (B). The luciferase acivity showed a significant decrease with the transfection of the plasmid mutated or deleted at four PBX2 binding sites. When PBX2 expression plasmid was co-transfected, the reporter activity significantly increased. Bars represent mean ± SE of at least three independent experiments. *Significant difference ($P < 0.05$).

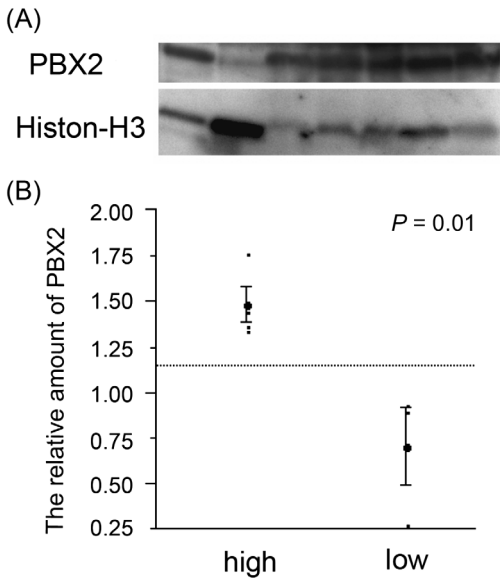

Fig. 4. Comparison of pre B cell leukemia transcription factor 2 (PBX2) protein level determined by Western blot (WB) and immunohistochemistry (IHC). (A) Amount of PBX2 and histone-H3 proteins in tissues from non-small cell lung cancer (NSCLC) was determined by WB. (B) The relative amount of PBX2 protein in cases with high and low expression by IHC.

Patient outcome. The 5-year disease-free survival (DFS) and overall survival (OS) rate of the patients was 67.9% and 73.6%, respectively. Tumors recurred in 79 patients: lung in 23, lymph node in 7, liver in 9, bone in 12, brain in 15, chest wall in 4, and other organs in 9. Seventy-one patients died due to the tumors.

Comparison of expression level of PBX2 protein at IHC and WB. To estimate the specificity of IHC for detection of PBX2 protein expression, WB analysis was performed. Nuclear fractions were obtained from seven cases with NSCLC showing various expression levels of PBX2 at IHC. Amount of PBX2 proteins in each fraction was quantified with WB (Fig. 4A). The samples with 'high' and 'low' staining intensity at IHC showed the high mean value and low mean value of PBX2 protein amounts, respectively (Fig. 4B). These results showed that IHC is a reliable method to determine the level of PBX2 protein expression in NSCLC. The signal intensities with pre-absorbed antibody dramatically decreased compared to those with non-absorbed antibody (Fig. 5).

PBX2 protein expression and clinicopathologic factors. One hundred (48.5%) and 106 (51.5%) cases showed 'high' and 'low' PBX2 nuclear expression, respectively (Fig. 6A,B). Patients with tumors of 'high' PBX2 expression showed the higher expression level of VCP and the higher rate of visceral pleural invasion than those of 'low' expression (Table 1).

Univariate and multivariate analysis for prognostic factors. Univariate analysis revealed the PBX2 and VCP expression, tumor size, histological differentiation, pT and pN classifications, and invasion to visceral pleura to be significant for both OS and DFS (Table 2). Microscopic vascular invasion significantly affected OS but not DFS, stage of pTNM classification

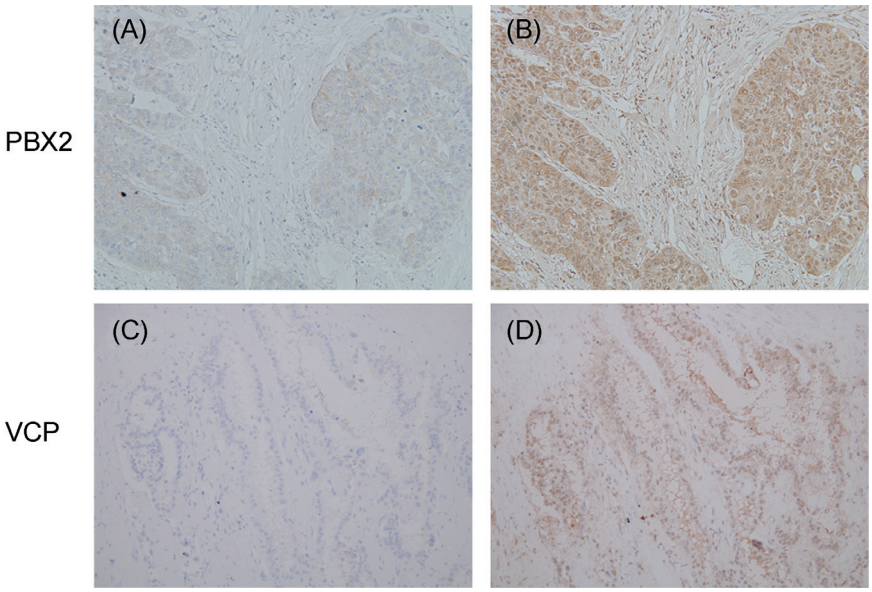

Fig. 5. Verification of the specificity of anti-pre B cell leukemia transcription factor 2 (PBX2) and anti-valosin-containing protein (VCP) antibodies. Immunohistochemistry with pre-absorbed anti-PBX2 (A), non-absorbed anti-PBX2 (B), pre-absorbed anti-VCP (C), non-absorbed anti-VCP (D) are shown. × 200.

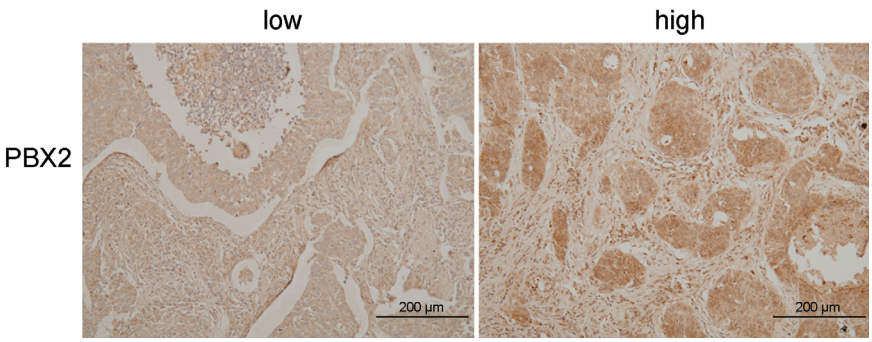

Fig. 6. Low and high expression of pre B cell leukemia transcription factor 2 (PBX2) by immunohistochemistry. Avidin-biotin-peroxidase complex method, ×200.

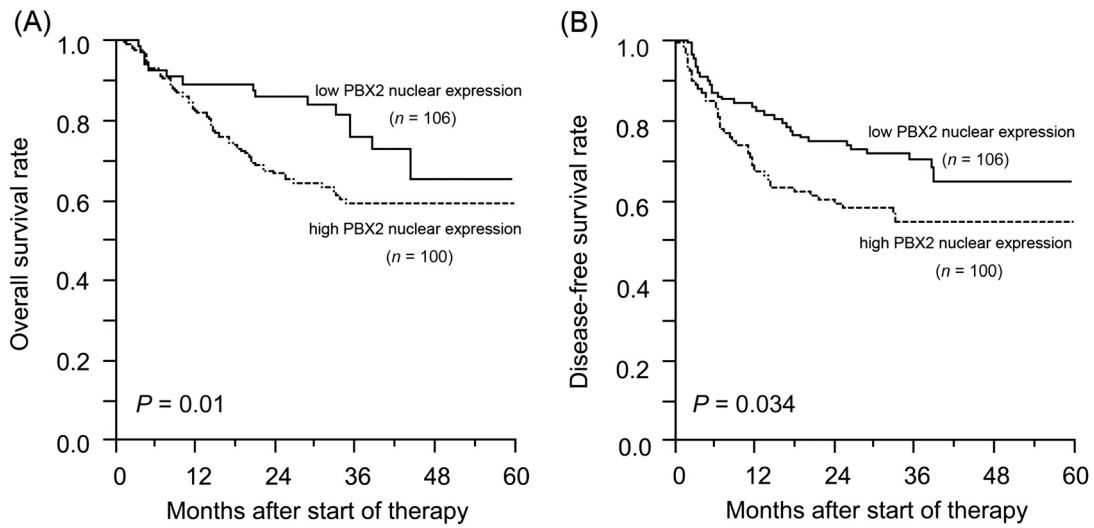

Fig. 7. Kaplan-Meier plots for overall (A) and disease-free survival (B) of patients with high and low expression non-small cell lung cancer.

doi: 10.1111/j.1349-7006.2009.01156.x

Table 1. Relationship between PBX2 expression and clinicopathologic factors in 206 patients with non-small cell lung cancer

Clinicopathological features	Category	Total number of patients $n = 206$	PBX2 high nuclear expression ($n = 100$)		PBX2 low nuclear expression ($n = 106$)		*P*-value
			No.	%	No.	%	
Age, mean ± SD			61.2 ± 8.1		59.4 ± 9.3		NS
Sex	1: Male	132	64	48.5%	68	51.5%	NS
	2: Female	74	36	48.6%	38	51.4%	
Tumor location	1: Left	71	37	52.1%	34	47.2%	NS
	2: Right	135	63	46.7%	72	53.3%	
Tumor size, mm	1: ≤30	80	38	47.5%	42	52.5%	NS
	2: >30	126	62	49.2%	64	50.8%	
Histological type	1: Adenocarcinoma	126	61	48.4%	65	51.6%	NS
	2: Squamous cell carcinoma	70	33	47.1%	37	52.9%	
	3: Others	10	6	60.0%	4	40.0%	
Histological differentiation	1: Well differentiated	94	48	51.1%	43	48.9%	NS
	2: Moderately differentiated	61	34	55.7%	38	44.3%	
	3: Poorly differentiated	51	18	35.3%	25	64.7%	
Manner of proliferation	1: Scirrhous	36	18	50.0%	18	50.0%	NS
	2: Intermediate	82	44	53.7%	38	46.3%	
	3: Medullary	88	38	43.2%	50	56.8%	
Vascular invasion	1: Absent	190	91	47.9%	99	52.1%	NS
	2: Present	16	9	56.3%	7	43.7%	
Lymphatic invasion	1: Absent	121	61	50.4%	60	49.6%	NS
	2: Present	85	39	45.9%	46	54.1%	
Visceral pleural invasion	1: Absent	168	75	44.6%	93	55.4%	< 0.05
	2: Present	38	25	65.8%	13	34.2%	
N(pTNM)	1: pN_0	174	80	46.0%	94	54.0%	NS
	2: pN_1	27	15	55.6%	12	44.4%	
	3: pN_2	5	5	100.0%	0	0.0%	
T(pTNM)	1: pT_1	119	58	48.7%	61	51.3%	NS
	2: pT_2	84	41	48.8%	43	51.2%	
	3: pT_3	3	1	33.3%	2	66.7%	
Stage	1: IA	103	47	45.6%	56	54.4%	NS
	2: IB	68	32	47.1%	36	52.9%	
	3: IIA	14	8	57.1%	6	42.9%	
	4: IIB	15	8	53.3%	7	46.7%	
	5: IIIA	6	5	83.3%	1	16.7%	
Smoking habit	1: Absent	72	32	44.4%	40	55.6%	NS
	2: Present	134	68	50.7%	66	49.3%	
Adjuvant therapy	1: Not performed	173	82	47.4%	91	52.6%	NS
	2: Performed	33	18	54.5%	15	45.5%	
VCP expression	1: High	137	74	54.0%	63	46.0%	< 0.05
	2: Low	69	26	37.7%	43	62.3%	

Abbreviations: PBX, pre B cell leukemia transcription factor; VCP, valosin-containing protein; NS, not significant.

affected DFS but not OS. Patients with 'high' PBX2 expression showed poorer OS and DFS than those with 'low' expression (Table 2, Fig. 7A,B). Multivariate analysis with factors proven to be significant in the univariate analysis revealed that VCP expression, visceral pleural invasion, and pT and pN classifications were independent prognostic factors for both OS and DFS, and vascular invasion to be significant for OS (Table 3). PBX2 nuclear expression was not significant in the multivariate analysis.

Prognostic significance of PBX expression in NSCLC patients with non-adjuvant therapy. Adjuvant therapy was not employed in 173 cases. Among them, 84 (48.6%) cases showed 'high' PBX2 nuclear expression. The correlation between PBX2 expression and clinicopathological factors is shown in Table 4. Patients with tumors of 'high' PBX2 expression showed the higher expression level of VCP than those of 'low' expression.

Among the patients without adjuvant therapy, those with 'high' expression of PBX2 and VCP showed poorer OS and DFS than those with 'low' expression (Fig. 8A–D). Univariate analysis revealed that PBX2 and VCP expression, tumor size, invasion to visceral pleura, pT and pN classifications, and tumor stage were significant unfavorable prognosticators for both DFS and OS (Table 5). Multivariate analysis with factors proven to be significant in the univariate analysis revealed that PBX2 expression and invasion to visceral pleura were independent prognostic factors for both OS and DFS, and VCP expression for OS (Table 6).

Prognostic significance of PBX and VCP expression in pTNM stage I cases. The number of patients in pTNM stage I was 171. Seventy-nine (46.2%) cases showed 'high' PBX2 nuclear expression. Patients with NSCLC at stage I showed favorable prognosis irrespective of PBX2 expression status (DFS: $P = 0.126$; OS: $P = 0.069$) (Fig. 9A,B). In contrast, patients with 'low' VCP

Table 2. Univariate analysis of clinicopathologic factors for disease-free and overall survival in 206 patients with non-small cell lung cancer

Factor	Category	No. patients	5-year disease-free survival rate (%)	*P*-value	5-year overall survival rate (%)	*P*-value
PBX2 nuclear expression	1: High	100	60.9%	<0.05	65.7%	<0.05
	2: Low	106	74.3%		80.9%	
VCP expression	1: High	137	63.3%	<0.05	66.9%	<0.005
	2: Low	69	76.8%		85.3%	
Age (y)	1: ≤60y	92	71.7%	NS	78.2%	NS
	2: >60y	114	64.6%		69.8%	
Sex	1: Male	132	68.9%	NS	73.4%	NS
	2: Female	74	66.2%		74.3%	
Tumor location	1: Left	71	66.2%	NS	70.4%	NS
	2: Right	135	68.7%		75.4%	
Tumor size, mm	1: ≤30	126	73.4%	<0.05	78.1%	<0.05
	2: >30	80	59.1%		66.7%	
Histological type	1: Adenocarcinoma	126	65.0%	NS	73.5%	NS
	2: Squamous cell carcinoma	70	76.1%		75.9%	
	3: Other	10	50.0%		60.0%	
Histological differentiation	1: Well differentiated	94	77.5%	<0.05	84.2%	<0.05
	2: Moderately differentiated	61	57.4%		66.7%	
	3: Poorly differentiated	51	64.8%		64.3%	
Pattern of connective tissue in tumor	1: Scirrhous	36	77.7%	NS	80.5%	NS
	2: Intermediate	82	63.8%		74.8%	
	3: Medullary	88	67.4%		69.6%	
Vascular invasion	1: Absent	190	69.9%	NS	75.7%	<0.05
	2: Present	16	43.7%		50.0%	
Lymphatic invasion	1: Absent	121	71.2%	NS	76.1%	NS
	2: Present	85	63.1%		70.2%	
Visceral pleural invasion	1: Absent	168	72.6%	<0.05	77.4%	<0.01
	2: Present	38	47.3%		57.0%	
N(pTNM)	1: pN_0	174	69.6%	<0.01*	75.9%	<0.005†
	2: pN_1	27	65.4%		69.2%	
	3: pN_2	5	20.0%		20.0%	
T(pTNM)	1: pT_1	119	75.2%	<0.05‡	82.0%	<0.05§
	2: pT_2	84	58.6%		65.8%	
	3: pT_3	3	33.3%		33.3%	
Stage	1: IA	103	68.9%	<0.05¶	70.9%	NS
	2: IB	68	50.0%	<0.05**	54.4%	
	3: IIA	14	57.1%		64.3%	
	4: IIB	15	53.3%		60.0%	
	5: IIIA	6	16.7%		16.7%	
Adjuvant therapy	1: Not performed	173	66.3%	NS	72.6%	NS
	2: Performed	33	75.7%		78.7%	
Smoking habit	1: Absent	72	63.3%	NS	71.8%	NS
	2: Present	134	70.3%		74.7%	

Abbreviations: PBX, pre B cell leukemia transcription factor; VCP, valosin-containing protein; NS, not significant.
* and † 1–2 versus 3.
‡ and § 1 versus 2–3.
¶ 1–2 versus 3–5.
**1 versus 2–5.

expression showed a better DFS and OS than those with 'high' expression (DFS: $P < 0.05$; OS: $P < 0.01$) (Fig. 9C,D).

Discussion

NSCLC is one of the most common human cancers, in which surgical removal of tumors still plays a major role in patient management, although it is usually followed with unfavorable prognosis.[22] Therefore, elucidation of factors affecting behavior of NSCLC is indispensable to improve prognosis. Previous studies have shown that performance status,[23] body weight loss, and gender[24] were independent prognostic factors for patients with NSCLC. Understanding the molecular mechanisms for development of NSCLC is expected to provide additional information for therapy. In this respect, abnormalities of several genes, such as p53[25] and k-ras,[26] were reported to be prognostic markers for NSCLC.

Patient characteristics found in the present series such as gender (male, 63.2%), age (mean, 64 years), pathologic stage (IA 51%, IB 32%, IIA 16%, IIB 8%, IIIA 3%), and 5-year survival rates (OS, 70.2%) were similar to those in the previous reports on NSCLC.[27–30] In addition, uni- and multivariate analyses in the present study revealed the prognostic significance of clinicopathologic factors such as visceral pleural invasion and lymph

doi: 10.1111/j.1349-7006.2009.01156.x

Table 3. Multivariate analysis of clinicopathologic factors for disease-free and overall survival in 206 patients with non-small cell lung cancer

Factor	Category	Relative risk	95% confidence interval	Chi-square value	*P*
Disease-free survival					
VCP expression	High Low	1.310	1.016–1.725	4.347	<0.05
pT (pTNM)	pT1 pT2–3	1.328	1.061–1.666	6.092	<0.05
pN (pTNM)	pN0–1 pN2	2.094	1.077–3.696	4.628	<0.05
Visceral pleural invasion	Present Absent	1.395	1.064–1.793	5.684	<0.05
PBX2 nuclear expression	High Low	1.147	0.905–1.458	1.292	0.255
Stage	IA + B IIA + B + IIIA	1.127	0.814–1.505	0.569	0.451
Overall survival					
VCP expression	high low	1.517	1.146–2.069	8.835	<0.01
pT (pTNM)	pT1 pT2–3	1.366	1.078–1.734	6.676	<0.05
pN (pTNM)	pN0–1 pN2	2.670	1.442–4.449	8.281	<0.01
Visceral pleural invasion	Present Absent	1.384	1.039–1.806	4.874	<0.05
Vascular invasion	Present Absent	1.456	0.983–2.036	3.541	0.060
PBX2 nuclear expression	High Low	1.176	0.914–1.518	1.588	0.208

Abbreviations: PBX, pre B cell leukemia transcription factor; VCP, valosin-containing protein; NS, not significant.

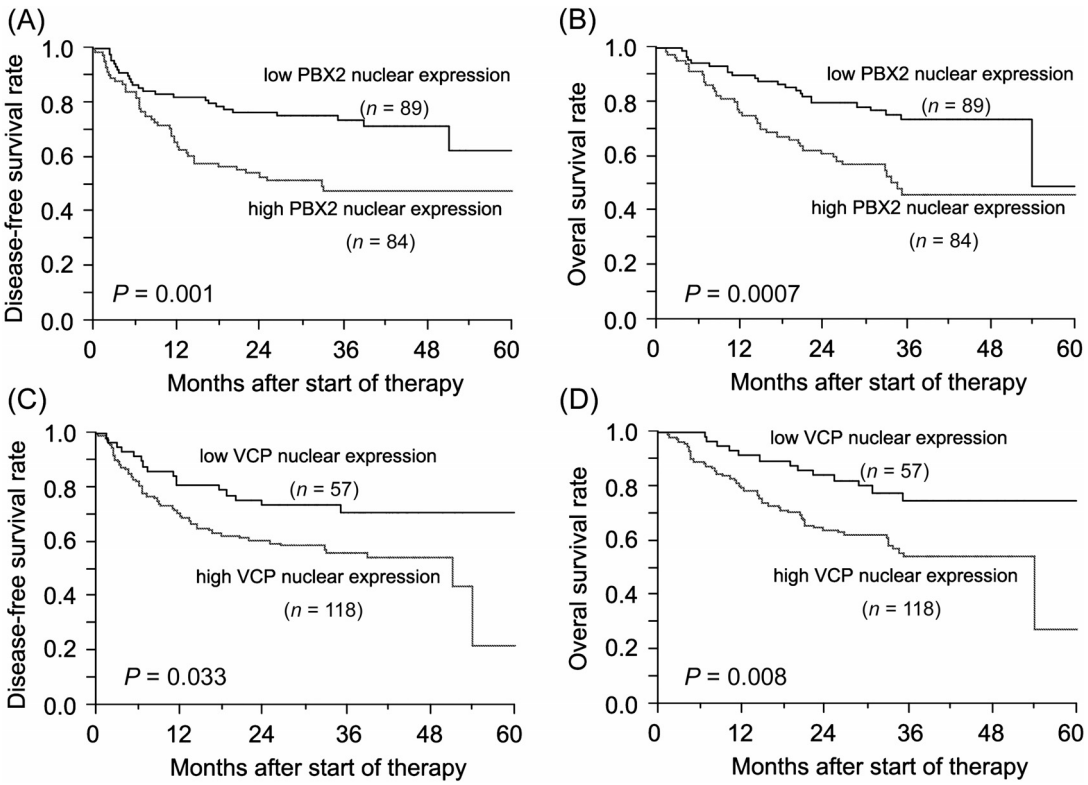

Fig. 8. Disease-free and overall survival (DFS and OS) rates of patients with high and low expression of pre B cell leukemia transcription factor 2 (PBX2) and valosin-containing protein (VCP) in non-small cell lung cancer patients without adjuvant therapy. Significant difference was observed between patients with high and low expression of PBX2 (A and B) and VCP (C and D) for DFS and OS.

Table 4. Relationship between PBX2 expression and clinicopathologic factors in 173 non-small cell lung cancer patients with non-adjuvant therapy

Clinicopathological features	Category	Total number of patients n = 173	PBX2 high nuclear expression (n = 84)		PBX2 low nuclear expression (n = 89)		P-value
			No.	%	No.	%	
Age, mean ± SD			62.1 ± 8.4		59.6 ± 9.1		NS
Sex	1: Male	110	50	45.5%	60	54.5%	NS
	2: Female	63	34	54.0%	29	46.0%	
Tumor location	1: Left	55	27	49.1%	28	50.9%	NS
	2: Right	118	57	48.3%	61	51.7%	
Tumor size, mm	1: ≤30	104	52	50.0%	52	50.0%	NS
	2: >30	69	32	46.4%	37	53.6%	
Histological type	1: Adenocarcinoma	105	52	49.5%	53	50.5%	NS
	2: Squamous cell carcinoma	59	26	44.1%	33	55.9%	
	3: Others	9	6	66.7%	3	33.3%	
Histological differentiation	1: Well differentiated	76	41	53.9%	35	46.1%	NS
	2: Moderately differentiated	61	29	47.5%	61	52.5%	
	3: Poorly differentiated	36	14	38.9%	36	61.1%	
Manner of proliferation	1: Scirrhous	31	15	48.4%	16	51.6%	NS
	2: Intermediate	70	37	52.9%	33	47.1%	
	3: Medullary	72	32	44.4%	40	55.6%	
Vascular invasion	1: Absent	158	77	48.7%	81	51.3%	NS
	2: Present	15	7	46.6%	6	53.4%	
Lymphatic invasion	1: Absent	101	51	50.5%	48	49.5%	NS
	2: Present	72	33	45.8%	39	54.2%	
Visceral pleural invasion	1: Absent	144	66	45.8%	78	54.2%	NS
	2: Present	29	18	62.1%	11	37.8%	
N (pTNM)	1: pN0	146	67	45.9%	79	54.1%	NS
	2: pN1	23	13	56.5%	10	43.5%	
	3: pN2	4	4	100.0%	0	0.0%	
T (pTNM)	1: pT1	99	49	49.5%	50	50.5%	NS
	2: pT2	71	34	47.9%	37	52.1%	
	3: pT3	3	1	33.3%	2	66.7%	
Stage	1: IA	86	39	45.3%	47	54.7%	NS
	2: IB	57	27	47.4%	30	52.6%	
	3: IIA	12	8	66.7%	4	33.3%	
	4: IIB	13	6	46.2%	7	53.8%	
	5: IIIA	5	4	80.0%	1	20.0%	
Smoking habit	1: Absent	62	28	45.2%	34	54.8%	NS
	2: Present	111	56	50.5%	55	49.5%	
VCP expression	1: High	118	63	53.4%	53	46.6%	<0.05
	2: Low	57	21	36.8%	36	63.2%	

Abbreviations: PBX, pre B cell leukemia transcription factor; VCP, valosin-containing protein; NS, not significant.

node metastasis, as reported previously.[27–30] These findings indicate that the results obtained from the present study are applicable to NSCLC worldwide.

The previous study showed that the level of VCP expression in tumor cells correlated with metastatic potential and poor prognosis in many kinds of cancers including NSCLC.[4,8–11] This was confirmed in the present study: VCP was an independent poor prognostic factor in NSCLC. PBX1, which belongs to the superfamily of homeodomain-containing proteins, proved to be a transcription factor enhancing VCP expression in breast cancer.[12] PBX2 gene encodes protein with extensive homology of 92% to PBX1. Despite the high homology, PBX1 and PBX2 may exhibit different affinities for the same target DNA sequences which are influenced by slight structural alterations due to their amino acid differences.[17] Knocked-down experimentation in this study revealed that the PBX2 expression showed a correlation with VCP expression in NSCLC cell lines. In contrast, no correlation was detected between expression of PBX1 and VCP. In addition, the staining intensity of NSCLC cells for PBX2 correlated with that of VCP in the clinical samples, suggesting that PBX2 transactivated VCP promoter in NSCLC. In fact, ChIP assay and luciferase reporter assay revealed that PBX2 enhanced the promoter activity of VCP gene through their recognition sites. In the breast cancer cell line MCF7, it was shown that PBX1 regulated the expression of VCP. These findings show that regulatory factors for VCP expression might be different by kind of tumors, that is, PBX1 in breast cancer and by PBX2 in NSCLC. Similar kinds of observation were made in other kinds of cells, that is, transactivation of platelet factor 4 gene was regulated by PBX1 in megakaryocytic cells, whereas by PBX2 in non-megakaryocytic cells.[31]

Univariate analysis showed that the higher level expression of PBX2 and VCP correlated with poor prognosis for patients with NSCLC. However, multivariate analysis revealed that the high level of VCP but not PBX2 expression was an independent factor for poor prognosis. Due to the strong correlation between expression levels of PBX2 and VCP as shown in the present

doi: 10.1111/j.1349-7006.2009.01156.x

Table 5. Univariate analysis of clinicopathologic factors for disease-free and overall survival in 173 non-small cell lung cnacer patients with non-adjuvant therapy

Factor	Category	No. patients	5-year disease-free survival rate (%)	*P*-value	5-year overall survival rate (%)	*P*-value
PBX2 nuclear expression	1: High	84	54.5%	<0.005	62.7%	<0.001
	2: Low	89	76.2%		80.6%	
VCP expression	1: High	118	60.9%	<0.05	66.0%	<0.01
	2: Low	57	75.4%		85.7%	
Age (years)	1: ≤60y		70.5%	NS	76.9%	NS
	2: >60y		60.6%		67.9%	
Sex	1: Male	110	67.1%	NS	72.5%	NS
	2: Female	63	63.5%		69.8%	
Tumor location	1: Left	55		NS		NS
	2: Right	118				
Tumor size, mm	1: ≤30	104	70.7%	NS	76.3%	<0.05
	2: >30	69	58.4%		65.6%	
Histological type	1: Adenocarcinoma	105	61.4%	NS	70.3%	NS
	2: Squamous cell carcinoma	59	74.9%		60.4%	
	3: Other	9	55.5%		55.5%	
Histological differentiation	1: Well differentiated	76	68.0%	NS	78.4%	NS
	2: Moderately differentiated	61	62.2%		62.2%	
	3: Poorly differentiated	36	63.3%		65.8%	
Pattern of connective tissue in tumor	1: Scirrhous	31	71.0%	NS	77.4%	NS
	2: Intermediate	70	57.8%		56.3%	
	3: Medullary	72	65.7%		68.4%	
Vascular invasion	1: Absent	158	67.4%	NS	74.2%	NS
	2: Present	13	46.2%		53.8%	
Lymphatic invasion	1: Absent	99	70.4%	NS	74.2%	NS
	2: Present	72	59.2%		67.6%	
Visceral pleural invasion	1: Absent	144	70.8%	<0.01	76.4%	<0.05
	2: Present	29	41.3%		53.9%	
N(pTNM)	1: pN_0	146	67.9%	<0.05*	74.6%	<0.01†
	2: pN_1	23	50.0%		44.4%	
	3: pN_2	4	25.0%		25.0%	
T(pTNM)	1: pT_1	99	73.2%	<0.05‡	80.3%	<0.01§
	2: pT_2	71	49.3%		50.7%	
	3: pT_3	3	33.3%		33.3%	
Stage	1: IA	86	75.0%	<0.05¶	80.7%	<0.05**
	2: IB	57	50.8%	<0.05††	49.1%	<0.01‡‡
	3: IIA	12	58.3%		58.3%	
	4: IIB	13	53.8%		46.2%	
	5: IIIA	5	20.0%		20.0%	
Smoking habit	1: Absent	72	60.7%	NS	67.2%	NS
	2: Present	134	68.7%		73.9%	

Abbreviations: PBX, pre B cell leukemia transcription factor; VCP, valosin-containing protein; NS, not significant.
* and † 1–2 versus 3.
‡ and § 1 versus 2–3.
¶ and ** 1–2 versus 3–5.
†† and ¶ 1 versus 2–5.

study, high PBX2 expression might not become an independent prognostic factor. Indeed the multivariate analysis with all factors but not VCP showed that the PBX2 was a significant factor for poor prognosis of NSCLC (data not shown). Among NSCLC with stage I disease, VCP was an independent prognostic factor for OS but not for DFS, suggesting that the VCP expression might not be involved in recurrence.

Subgroup analysis excluding cases with adjuvant therapy revealed that PBX2 expression but not VCP expression was an independent poor prognostic factor both in DFS and OS. As a transcription factor, PBX2 may regulate the expression of various factors other than VCP. Some unknown targets for PBX2 might play important roles for prognosis of NSCLC without adjuvant therapies.

In conclusion, PBX2 expression correlated with VCP level, indicating that PBX2 works as a transcription factor enhancing VCP expression. The present study clearly demonstrated that higher nuclear expression of PBX2 was a poor prognostic factor in NSCLC as revealed by univariate analysis. Besides the enhancement of VCP expression, functional significance of PBX2 remains clarified in this study. Further studies on PBX2 function in NSCLC would be necessary to evaluate whether PBX2 could be a target in the treatment of NSCLC.

Acknowledgments

This work was supported in part by the International Collaborative Project of National Natural Science Foundation of China (No. 30801382).

Table 6. Multivariate analysis of clinicopathologic factors for disease-free and overall survival in 173 non-small cell lung cancer patients with non-adjuvant therapy

Factor	Category	Relative risk	95% confidence interval	Chi-square Value	*P*
Disease-free survival					
PBX2 nuclear expression	High Low	1.446	1.113–1.898	7.661	<0.01
VCP expression	High Low	1.292	0.978–1.753	3.235	0.072
pT (pTNM)	pT1 pT2–3	1.586	0.942–2.815	1.223	0.082
pN (pTNM)	pN0–1 pN2	1.498	0.696–2.784	3.006	0.268
Visceral pleural invasion	Present Absent	1.489	1.111–1.952	6.811	<0.01
Stage	IA + B IIA + B + IIIA	1.396	0.917–2.037	2.493	0.114
Overall survival					
PBX2 nuclear expression	High Low	1.472	1.119–1.957	7.707	<0.01
VCP expression	High Low	1.443	1.070–2.018	5.892	<0.05
pT (pTNM)	pT1 pT2–3	1.626	0.942–2.954	3.048	0.114
pN (pTNM)	pN0–1 pN2	1.857	0.854–3.521	2.581	0.114
Visceral pleural invasion	Present Absent	1.479	1.104–1.982	6.518	<0.05
Stage	IA + B IIA + B + IIIA	1.340	0.863–1.983	1.786	0.181

Abbreviations: PBX, pre B cell leukemia transcription factor; VCP, valosin-containing protein; NS, not significant.

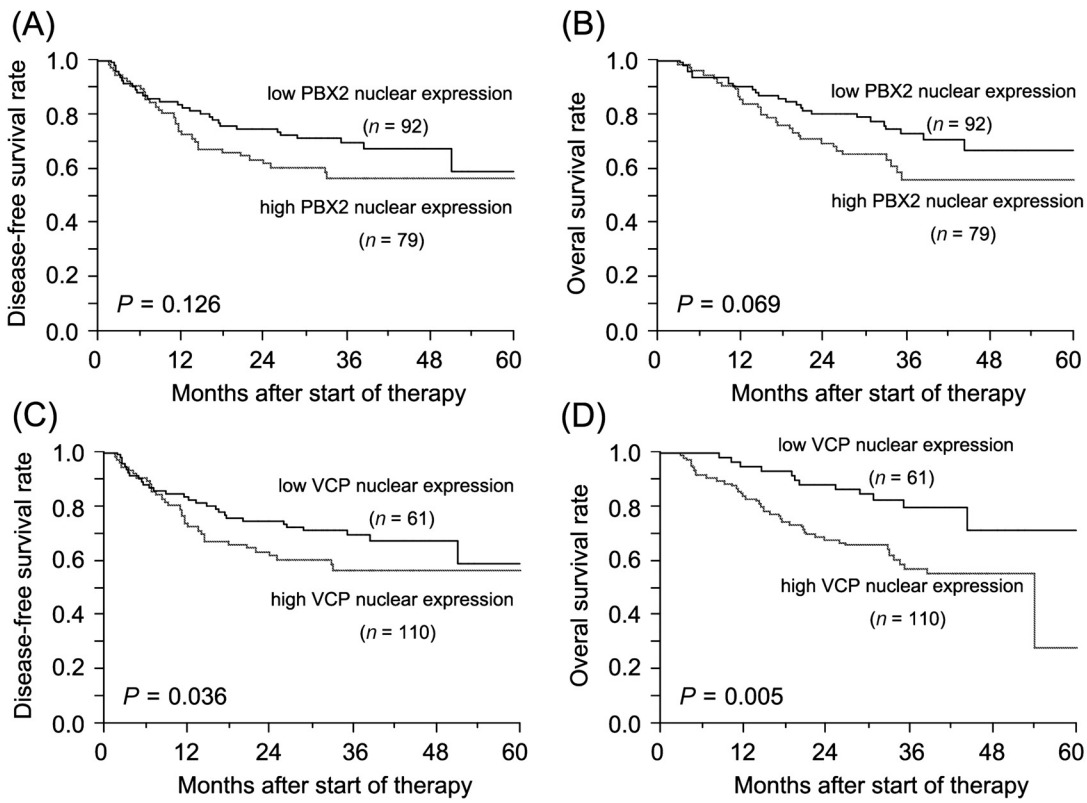

Fig. 9. Disease-free and overall survival (DFS and OS) rates of patients with high and low expression of pre B cell leukemia transcription factor 2 (PBX2) and VCP at Stage I disease of pathologic tumor-node-metastasis (pTNM) classification. Significant difference in survival was not observed between patients with high and low PBX2 expression (A and B), but significant difference was observed between high and low VCP expression for DFS and OS (C and D).

doi: 10.1111/j.1349-7006.2009.01156.x

References

1 Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of eighteen major cancers in 1985. *Int J Cancer* 1993; **54**: 594–606.
2 Gridelli C, Rossi A, Galetta D *et al*. Italian clinical research in non-small-cell lung cancer. *Ann Oncol* 2005; **16**: 110–5.
3 Pirozynski M. 100 years of lung cancer. *Respir Med* 2006; **100**: 2073–84.
4 Yamamoto S, Tomita Y, Hoshida Y *et al*. Expression level of valosin-containing protein (p97) is correlated with progression and prognosis of non-small-cell lung carcinoma. *Ann Surg Oncol* 2004; **11**: 697–704.
5 Asai T, Tomita Y, Nakatsuka S *et al*. VCP (p97) regulates NFkappaB signaling pathway, which is important for metastasis of osteosarcoma cell line. *Jpn J Cancer Res* 2002; **93**: 296–304.
6 Dai RM, Chen E, Longo DL, Gorbea CM, Li CC. Involvement of valosin-containing protein, an ATPase Co-purified with IkappaBalpha and 26 S proteasome, in ubiquitin-proteasome-mediated degradation of IkappaBalpha. *J Biol Chem* 1998; **273**: 3562–73.
7 Dai RM, Li CC. Valosin-containing protein is a multi-ubiquitin chain-targeting factor required in ubiquitin-proteasome degradation. *Nat Cell Biol* 2001; **3**: 740–4.
8 Yamamoto S, Tomita Y, Nakamori S *et al*. Elevated expression of valosin-containing protein (p97) in hepatocellular carcinoma is correlated with increased incidence of tumor recurrence. *J Clin Oncol* 2003; **21**: 447–52.
9 Yamamoto S, Tomita Y, Hoshida Y *et al*. Expression level of valosin-containing protein is strongly associated with progression and prognosis of gastric carcinoma. *J Clin Oncol* 2003; **21**: 2537–44.
10 Yamamoto S, Tomita Y, Hoshida Y *et al*. Expression of valosin-containing protein in colorectal carcinomas as a predictor for disease recurrence and prognosis. *Clin Cancer Res* 2004; **10**: 651–7.
11 Yamamoto S, Tomita Y, Hoshida Y *et al*. Expression level of valosin-containing protein (VCP) as a prognostic marker for gingival squamous cell carcinoma. *Ann Oncol* 2004; **15**: 1432–8.
12 Qiu Y, Tomita Y, Zhang B, Nakamichi I, Morii E, Aozasa K. Pre-B-cell leukemia transcription factor 1 regulates expression of valosin-containing protein, a gene involved in cancer growth. *Am J Pathol* 2007; **170**: 152–9.
13 Kamps MP, Murre C, Sun XH, Baltimore D. A new homeobox gene contributes the DNA binding domain of the t (1; 19) translocation protein in pre–B ALL. *Cell* 1990; **60**: 547–55.
14 Nourse J, Mellentin JD, Galili N *et al*. Chromosomal translocation t (1; 19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. *Cell* 1990; **60**: 535–45.
15 Dedera DA, Waller EK, LeBrun DP *et al*. Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. *Cell* 1993; **74**: 833–43.
16 Mertelsmann R. Application of basic science to hematopoiesis and treatment of disease. *Ann Oncol* 1992; **3**: 269–72.
17 Monica K, Galili N, Nourse J, Saltman D, Cleary ML. PBX2 and PBX3, new homeobox genes with extensive homology to the human proto-oncogene PBX1. *Mol Cell Biol* 1991; **11**: 6149–57.
18 Kamps MP, Look AT, Baltimore D. The human t (1; 19) translocation in pre–B ALL produces multiple nuclear E2A–Pbx1 fusion proteins with differing transforming potentials. *Genes Dev* 1991; **5**: 358–68.
19 Phelan ML, Rambaldi I, Featherstone MS. Cooperative interactions between Hox and Pbx proteins mediated by a conserved peptide motif. *Mol Cell Biol* 1995; **15**: 3989–97.
20 Brambilla E, Travis WD, Colby TV, Corrin B, Shimosato Y. The new World Health Organization classification of lung tumours. *Eur Respir J* 2001; **18**: 1059–68.
21 Sobin LH, Wittekind CH. *TNM Classification of Malignant Tumours* (ed 6). New York, NY: Wiley-Liss 2002.
22 Mountain CF. Revisions in the international system for staging lung cancer. *Chest* 1997; **111**: 1710–7.
23 Buccheri G, Ferrigno D, Tamburini M. Karnofsky and ECOG performance status scoring in lung cancer: a prospective, longitudinal study of 536 patients from a single institution. *Eur J Cancer* 1996; **32A**: 1135–41.
24 Palomares MR, Sayre JW, Shekar KC, Lillington LM, Chlebowski RT. Gender influence on weight-loss pattern and survival of nonsmall cell lung carcinoma patients. *Cancer* 1996; **78**: 2119–26.
25 Quinlan DC, Davidson AG, Summers CL, Warden HE, Doshi HM. Accumulation of p53 protein correlates with a poor prognosis in human lung cancer. *Cancer Res* 1992; **52**: 4828–31.
26 Slebos RJ, Kibbelaar RE, Dalesio O *et al*. K-ras oncogene activation as a prognostic marker in adenocarcinoma of the lung. *N Engl J Med* 1990; **323**: 561–5.
27 Suzuki K, Nagai K, Yoshida J *et al*. Conventional clinicopathologic prognostic factors in surgically resected nonsmall cell lung carcinoma. A comparison of prognostic factors for each pathologic TNM stage based on multivariate analyses. *Cancer* 1999; **86**: 1976–84.
28 Fry WA, Phillips JL, Menck HR. Ten-year survey of lung cancer treatment and survival in hospitals in the United States: a national cancer data base report. *Cancer* 1999; **86**: 1867–76.
29 Shimizu K, Yoshida J, Nagai K *et al*. Visceral pleural invasion is an invasive and aggressive indicator of non-small cell lung cancer. *J Thorac Cardiovasc Surg* 2005; **130**: 160–5.
30 Sakao Y, Miyamoto H, Yamazaki A *et al*. Prognostic significance of metastasis to the highest mediastinal lymph node in non-small cell lung cancer. *Ann Thorac Surg* 2006; **81**: 292–7.
31 Okada Y, Nagai R, Sato T *et al*. Homeodomain proteins and PBXs regulate the lineage-specific transcription of the platelet factor 4 gene. *Blood* 2003; **101**: 4748–56.