Expression of CUB domain containing protein (CDCP1) is correlated with prognosis and survival of patients with adenocarcinoma of lung

Jun-ichiro Ikeda,[1] Tomofumi Oda,[1,2] Masayoshi Inoue,[2] Takamasa Uekita,[3] Ryuichi Sakai,[3] Meinoshin Okumura,[2] Katsuyuki Aozasa[1] and Eiichi Morii[1,4]

Departments of [1]Pathology and [2]Thoracic Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka, and [3]Growth Factor Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo, Japan

(Received September 29, 2008/Revised November 4, 2008/Accepted November 19, 2008/Online publication December 11, 2008)

CUB domain containing protein (CDCP1), a transmembrane protein with intracellular tyrosine residues which are phosphorylated upon activation, is supposed to be engaged in proliferative activities and resistance to apoptosis of cancer cells. Expression level of CDCP1 was examined in lung adenocarcinoma, and its clinical implications were evaluated. CDCP1 expression was immunohistochemically examined in lung adenocarcinoma from 200 patients. Staining intensity of cancer cells was categorized as low and high in cases with tumor cells showing no or weak and strong membrane staining, respectively. MIB-1 labeling index was also examined. There were 113 males and 87 females with median age of 63 years. Stage of disease was stage I in 144 cases (72.0%), II in 19 (9.5%), and III in 37 (18.5%). Sixty of 200 cases (30.0%) were categorized as CDCP1-high, and the remaining as CDCP1-low. Significant positive correlation was observed between CDCP1-high expression and relapse rate ($P < 0.0001$), poor prognosis ($P < 0.0001$), MIB-1 labeling index ($P < 0.0001$), and occurrence of lymph node metastasis ($P = 0.0086$). There was a statistically significant difference in disease-free survival (DFS) ($P < 0.0001$) and overall survival (OS) rates ($P < 0.0001$) between patients with CDCP1-high and CDCP1-low tumors. Univariate analysis showed that lymph node status, tumor stage, and CDCP1 expression were significant factors for both OS and DFS. Multivariate analysis revealed that only CDCP1 expression was an independent prognostic factor for both OS and DFS. CDCP1 expression level is a useful marker for prediction of patients with lung adenocarcinoma (*Cancer Sci* 2009; 100: 429–433).

Introduction

Since 1985 lung cancer has been the most common cause of cancer death in the world.[1] Non-small cell lung cancer (NSCLC) comprises 75–85% of all lung cancers, and approximately two-thirds of NSCLC patients have advanced stages at diagnosis. Despite the advances in the methods for detection and treatment of lung cancer, prognosis of NSCLC patients still remains unfavorable. Therefore, it is important to clarify the mechanism of tumor biology, and establishment of effective therapeutic modalities is essential to improve the prognosis in NSCLC. Previous studies accumulated information regarding the factors influencing prognosis in NSCLC. They include clinical, pathological, and molecular factors.

CUB domain containing protein (CDCP1) was originally identified as an epithelial tumor antigen by comparisons of molecules expressed in lung cancer cell lines and normal lung tissues.[2] CDCP1 is a transmembrane protein with three extracellular CUB domains, which are important for cell–cell interactions, and intracellular tyrosine residues which are phosphorylated upon activation.[2–7] Previously, we reported the epigenetic regulation of CDCP1 expression in the cell lines derived from various malignancies and clinical samples of breast cancer.[8,9] The CDCP1 expression level correlated with proliferative activities of breast cancer cells in the clinical samples.[8] Very recently, CDCP1 was reported to protect cells from anoikis, a form of apoptosis triggered by the loss of cell survival signals generated from interaction of cells with the extracellular matrix.[10] The knocked-down expression of CDCP1 by RNA interference abolished *in vitro* colony formation and *in vivo* metastatic abilities of lung adenocarcinoma cell line A549.[10] These findings showed that CDCP1 is required for protection of cells from anoikis, and suggest an important role of CDCP1 for tumorigenesis and metastasis, at least in cell lines. In the present study, CDCP1 expression was immunohistochemically examined in clinical samples from lung adenocarcinoma, and its clinical implications were evaluated.

Materials and Methods

Patients and tissue samples. Two hundred patients who underwent surgery for lung adenocarcinoma at Osaka University Hospital during the period from January 1993 to January 2004 were examined. Clinicopathological findings in these 200 patients are summarized in Table 1. There were 113 men and 87 women with ages ranging from 33 to 82 years (median, 63). Resected specimens were macroscopically examined to determine the location and size of the tumors. The size of the main tumor ranged from 8 to 70 mm (median, 24.5). The histological stage was determined according to the 6th edition of the Union International Contre le Cancer – TNM staging system.[11] Histologic specimens were fixed in 10% formalin and routinely processed for paraffin-embedding. Paraffin-embedded specimens were stored in the dark room in the Department of Pathology of Osaka University Hospital at room temperature, and were sectioned at 4-μm thickness at the time of staining. In some cases, total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA, USA) with DNase I treatment. 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- to 12-month intervals. The follow-up period for survivors ranged from 5 to154 months (median, 63). The study was approved by the ethical review board of the Graduate

This work is original, and contains no materials previously presented in any reports and publications.

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

doi: 10.1111/j.1349-7006.2008.01066.x

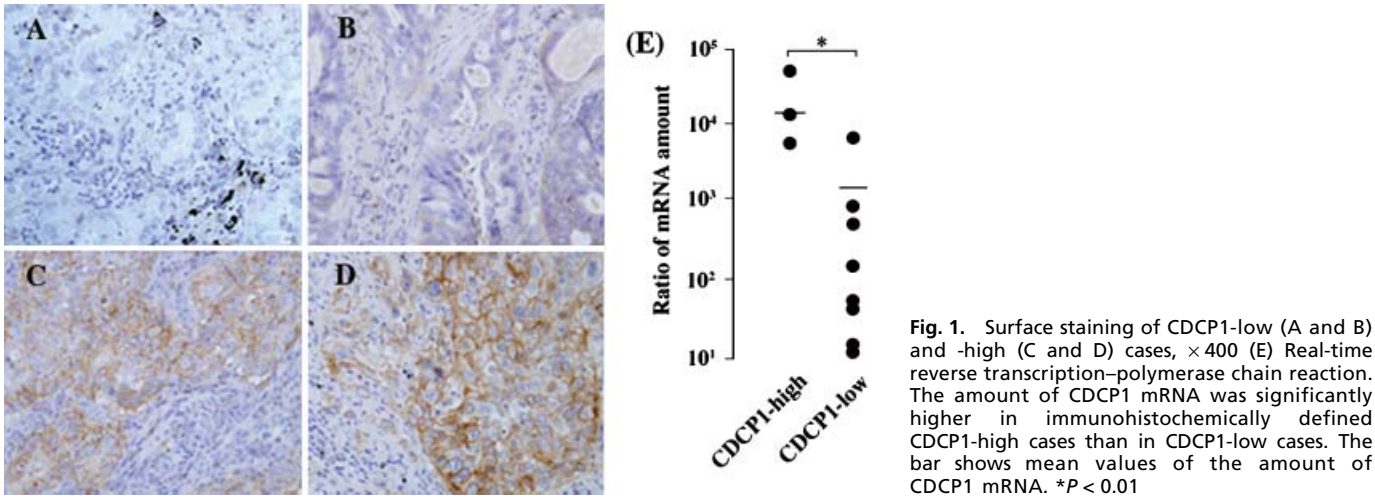

Fig. 1. Surface staining of CDCP1-low (A and B) and -high (C and D) cases, ×400 (E) Real-time reverse transcription–polymerase chain reaction. The amount of CDCP1 mRNA was significantly higher in immunohistochemically defined CDCP1-high cases than in CDCP1-low cases. The bar shows mean values of the amount of CDCP1 mRNA. *$P < 0.01$

Table 1. Summary of characteristics in 200 pulmonary adenocarcinoma patients

Sex	Number of patients
Male	113
Female	87
Tumor size (cm)	
≥5	12
<5	187
Lymph node metastasis	
N0	159
N1	8
N2	29
N3	4
Stage	
I	144
II	19
III	37
Recurrence	
Positive	60
Negative	140
Prognosis	
Dead	41
Alive (with recurrence)	24
Alive (with no recurrence)	135

School of Medicine, Osaka University. Informed consent was obtained from each patient.

Immunohistochemistry for CDCP1, phosphorylated CDCP1 and Ki-67. CDCP1 expression was immunohistochemically examined with use of anti-CDCP1 (Abcam Ltd, Cambridge, UK) and antiphosphorylated CDCP1 antibody. The antiphosphorylated CDCP1 antibody recognizes CDCP1 phosphorylated at Tyr734 and can be used for immunostaining on paraffin-embedded sections.[10,12] The proliferative activity of cancer cells was examined with monoclonal antibody MIB-1 (Immunotech, Marseilles, France), recognizing the proliferation-associated antigen Ki-67. After antigen retrieval with Pascal pressurized heating chamber (Dako, Glostrup, Denmark), the sections were incubated with anti-CDCP1, phosphorylated CDCP1 antibody and MIB-1, diluted at ×200, ×400 and ×100, respectively. Then, the sections were treated with biotin-conjugated antigoat IgG (Zymed, San Francisco, CA, USA) for CDCP1 staining, or with biotin-conjugated antimouse IgG (Dako) for phosphorylated CDCP1 and MIB-1 staining. After washing, the sections were incubated with the peroxidase-conjugated biotin-avidin complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA). diaminobenzidine (Vector Laboratories) was used as a chromogen. As the negative control, staining was carried out in the absence of a primary antibody. Stained sections were evaluated independently by two pathologists (JI and EM). Generally, CDCP1 expression levels varied among tumor cells in the same case. Staining intensity of tumor cells was divided into four categories; tumor cells with no (representative field was demonstrated in Fig. 1A), weak (Fig. 1B), moderate (Fig. 1C), or strong (Fig. 1D) membrane staining. The intensity of CDCP1 expression in each case was defined by the major population of staining as follows: cases with tumor cells showing no or weak membrane staining were categorized as CDCP1-low, and those showing moderate or strong membrane staining as CDCP1-high. The MIB-1 labeling index was defined as the percentage of stained nuclei per 1000 cells. The cases were divided into MIB-1-high and MIB-1-low groups using the median as cut-off value.

Quantification of mRNA by real-time reverse transcription-polymerase chain reaction (RT-PCR). To evaluate the specificity of CDCP1 immunostaining, expression level of CDCP1 at mRNA and protein levels was compared. For this, fresh frozen materials were available in 13 of the 200 cases. Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA, USA) with DNase I treatment. Two micrograms of total RNA was subjected to reverse transcription using Superscript III (Invitrogen, Carlsbad, CA, USA). The mRNA levels for CDCP1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were verified using TaqMan Gene Expression Assays (Hs00224587_m1 and 4310884E, respectively; Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. The amount of CDCP1 mRNA was normalized to that of GAPDH mRNA.

Statistical analysis. Statistical analyses were performed using StatView software (SAS Institute Inc., Cary, NC, USA). The Chi-square and Fisher's exact probability test were used to analyze the correlation between CDCP1 expression and clinicopathological factors in pulmonary adenocarcinoma. Kaplan-Meier methods were used to calculate overall survival (OS) and disease-free survival (DFS) rate, and differences in survival curves were evaluated with the log-rank test. Cox's proportional hazards regression model with a stepwise manner was used to analyze the independent prognostic factors. The *P*-values of less than 0.05 were considered to be statistically significant.

Results

Tumor stages in the present patients were: stage I in 144 patients (72.0%); II in 19 patients (9.5%); and III in 37 patients (18.5%). The histological types of tumors were: bronchioloalveolar

doi: 10.1111/j.1349-7006.2008.01066.x

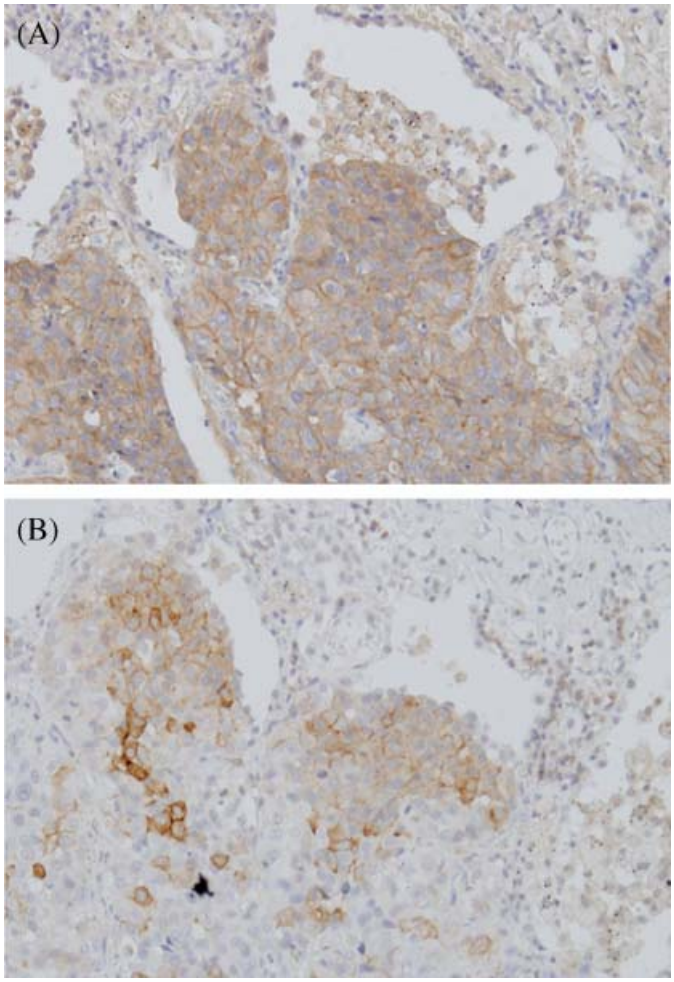

Fig. 2. Localization of phosphorylated CDCP1 in lung adenocarcinoma. Stained cells with anti-CDCP1 antibody (A), and antiphosphorylated CDCP1 antibody (B). Among the CDCP1-positive tumor cells, peripheral areas of tumor cell nests were stained with antiphophorylated CDCP1 antibody, × 400.

(62 patients, 31.0%); papillary (48 patients, 24.0%); or mixed bronchioloalveolar and papillary adenocarcinoma (90 patients, 45.0%). The 5-year DFS and OS was 78.7% and 80.6%, respectively. Tumors recurred in 60 patients. Of these, 38 patients died due to the tumors.

To evaluate the specificity of immunohistochemical staining for CDCP1 expression, quantitative real-time RT-PCR was performed: expression levels of CDCP1 at protein and mRNA level was compared in 13 cases (3 CDCP1-high and 10 CDCP1-low cases at immnohistochemical results). The amount of CDCP1 mRNA was significantly higher in cases with CDCP1-high expression at immunohistochemistry than those with CDCP1-low expression ($P < 0.01$, Fig. 1E). These results showed that the immunohistochemical evaluation is a reliable method for evaluation of CDCP1 expression.

Immunohistochemical detection of CDCP1 expression was carried out in 200 lung adenocarcinoma tissues. Sixty of 200 cases (30.0%) were categorized as CDCP1-high, and the remaining as CDCP1-low. Representative staining results were illustrated in Fig. 1(A–D).

Intracellular tyrosine residues of CDCP1 are known to be phosphorylated upon activation *in vitro*. To examine the localization of activated CDCP1, 43 cases of CDCP1-high lung adenocarcinoma tissues were stained with antiphosphorylated CDCP1. Phosphorylated CDCP1 was detected only in a small portion of CDCP1-expressing cells (Fig. 2 A and 2B), which

Table 2. Correlation between CDCP1 expression and clinicopathological parameters

	CDCP1 expression		*P*
	Low	High	
Tumor size (cm)			
≥5	7	5	
<5	133	55	0.3630
Lymph node metastasis			
N0	120	39	
N1	3	5	
N2	15	14	
N3	2	2	0.0086
Stage			
I	110	34	
II	11	8	
III	19	18	0.0059
MIB-1 labeling index			
≥5%	56	45	
<5%	84	15	<0.0001
Recurrence			
Positive	23	37	
Negative	117	23	<0.0001
Prognosis			
Dead	17	24	
Alive (with recurrence)	10	14	
Alive (with no recurrence)	113	22	<0.0001

appeared to be localized to the peripheral areas of tumor cell nests. Cells without CDCP1 expression did not show any phosphorylated CDCP1 signals, indicating the specificity of the antiphophorylated CDCP1 antibody. Phosphorylated CDCP1 was detected in 19 out of 43 cases: any significant clinicopathological differences were not observed between cases with and without phosphorylated CDCP1.

Correlation of CDCP1 expression with the clinicopathological features was evaluated. Significant positive correlation was observed between CDCP1-high expression and relapse rate ($P < 0.0001$), poor prognosis ($P < 0.0001$), MIB-1 labeling index ($P < 0.0001$), and occurrence of lymph node metastasis ($P = 0.0086$). Other parameters including tumor size and stage did not correlate with CDCP1 expression (Table 2). There was a statistically significant difference in DFS rates ($P < 0.0001$) and OS rates ($P < 0.0001$) between patients with CDCP1-high and CDCP1-low tumors (Fig. 3).

Univariate analysis showed that lymph node status, tumor stage, and CDCP1 expression were significant factors for both OS and DFS (Table 3). The multivariate analysis revealed that only CDCP1 expression was an independent prognostic factor for both OS and DFS.

Discussion

Patient characteristics such as the gender (male preponderance), age distribution (median age, 6th decades of life), and 5-year OS of approximately 80% in the present study were similar to those in a previous report on the lung adenocarcinoma.[13] In addition, the univariate analysis showed the prognostic significance of occurrence of lymph node metastasis and stage of disease, as reported previously.[13] These findings indicate that the results obtained from the present cases are commonly applicable.

Among the clinicopathological factors examined, high CDCP1 expression level correlated with increased occurrence of lymph node metastasis and tumor relapse. A previous study using the lung adenocarcinoma cell lines indicated a significant role of CDCP1 for anchorage-independent growth of tumor cells.[10]

Table 3. Univariate and multivariate analyses of prognostic factors for overall and disease-free survivals

	Overall survival				Disease-free survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Tumor size	1.07 (0.79–1.43)	0.672			1.11 (0.86–1.45)	0.425		
Lymph node status	2.34 (1.77–3.08)	<0.001	1.46 (0.85–2.50)	0.167	2.40 (1.82–3.17)	<0.001	1.43 (0.84–2.40)	0.182
Stage	2.64 (1.90–3.69)	<0.001	1.63 (0.87–3.06)	0.128	2.77 (1.99–3.86)	<0.001	1.74 (0.95–3.20)	0.074
MIB-1 labeling index	1.46 (0.78–2.74)	0.235			1.44 (0.77–2.69)	0.250		
CDCP1 expression	4.11 (2.18–7.75)	<0.001	2.89 (1.51–5.54)	0.001	4.32 (2.31–8.08)	<0.001	3.04 (1.60–5.80)	<0.001

HR, hazard ratio; CI, confidence interval.

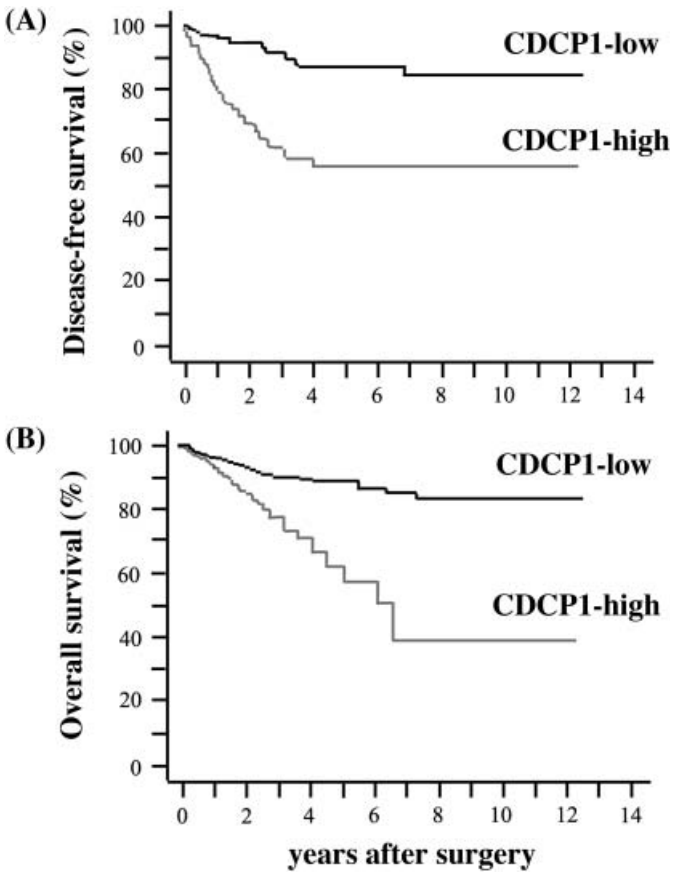

Fig. 3. Kaplan-Meier plots of disease-free (A) and overall survival (B) of patients.

The knocked-down expression of CDCP1 abolished ability of *in vitro* colony formation in the A549 lung adenocarcinoma cell line.[10] In addition, when injected into nude mice, the number of metastatic nodules was low in CDCP1-knocked down A549 cells as compared to parental A549 cells.[10] Taken together with the present results, CDCP1 appeared to play important roles for metastatic and tumorigenic potentials of lung adenocarcinoma not only in cell lines but also in clinical samples.

High CDCP1 expression was correlated with MIB-1 labeling index. Since the monoclonal antibody MIB-1 recognizes Ki-67 antigen that is expressed in cells during the cell cycle, except at the G0 phase, it can be applied to evaluate the proliferative activities of cells. Previously, we showed the positive correlation of CDCP1 expression with MIB-1 labeling index in breast cancer cells.[8] These findings indicate that CDCP1 expression level reflects a proliferative activity of cancer cells.

Intracellular tyrosine residues of CDCP1 are known to be phosphorylated upon activation, and the level of tyrosine phosphorylation is associated with the capacity for anchorage independence in A549 cells.[10] Immunohistochemically, phosphorylated CDCP1 was found to be localized to the peripheral areas of tumor cell nests. Lung adenocarcinoma cells often show bronchioalveolar growth in the periphery of the cancer tissues, but such portions were almost negative for phosphorylated CDCP1 expression. Phosphorylated CDCP1 was mostly present in the tumor cells expanding to the surrounding normal tissues. This was consistent with the previous report that phosphorylated CDCP1 is localized in the invasive front of gastric cancer.[12] Therefore, phosphorylated CDCP1 may play some roles for tumor invasion, in addition to anchorage independence. The staining for phosphorylated and non-phosphorylated CDCP1 demonstrated that most tumor cells expressed CDCP1 as a non-phosphorylated form. This was consistent with the report by Brown *et al*. that the phosphorylation of CDCP1 is dynamically balanced by Src-family kinase and phosphotyrosine phosphatase activities, yielding low equilibrium phosphorylation.[6] CDCP1 contains three extracellular CUB domains, which might be involved in cell adhesion or interaction with the extracellular matrix.[2–7] Non-phosphorylated CDCP1 may function as an adhesion molecule.

Multivariate analysis revealed the high expression of CDCP1 to be an independent factor for poor prognosis for patients with lung adenocarcinoma. Benes *et al*. reported that Src, which mediates proliferation signals in cancers, forms a complex with phosphorylated CDCP1.[7] These findings indicate that overexpression of CDCP1 could stimulate tumor growth, explaining why prognosis of patients with CDCP1-high tumors is worse than that with CDCP1-low tumors.

In conclusion, high CDCP1 expression is an independent factor for poor prognosis of patients with lung adenocarcinoma. Further studies will be necessary to elucidate whether CDCP1 expression could be a useful marker for prediction of prognosis in other types of cancers. CDCP1 could be a molecular target for cancer therapy.

Acknowledgments

The authors thank Ms. Megumi Sugano and Ms. Takako Sawamura for their technical assistance. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, and from the Osaka Cancer Research Foundation

doi: 10.1111/j.1349-7006.2008.01066.x

References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global Cancer Statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Scherl-Mostageer M, Sommergruber W, Abseher R, Hauptmann R, Ambros P, Shweifer N. Identification of a novel gene, CDCP1, overexpressed in human colorectal cancer. *Oncogene* 2001; **20**: 4402–8.
3 Hooper JD, Zijlstra A, Aimes RT *et al*. Subtractive immunization using highly metastatic human tumor cells identifies SIMA135/CDCP1, a 135 kDa cell surface phosphorylated glycoprotein antigen. *Oncogene* 2003; **22**: 1783–94.
4 Conze T, Lammers R, Kuci S *et al*. CDCP1 is a novel marker for hematopoietic stem cells. *Ann NY Acad Sci* 2003; **996**: 222–6.
5 Buhring H-J, Kuci S, Conze T *et al*. CDCP1 identifies a broad spectrum of normal and malignant stem/progenitor cell subsets of hematopoietic and nonhematopoietic origin. *Stem Cells* 2004; **22**: 334–43.
6 Brown TA, Yang TM, Zaitsevskaia T *et al*. Adhesion or plasmin regulates tyrosine phosphorylation of a novel membrane glycoprotein p80/gp140/CUB domain-containing protein 1 in epithelia. *J Biol Chem* 2004; **279**: 14772–83.
7 Benes CH, Wu N, Elia AEH, Dharia T, Cantley LC, Soltoff SP. The C2 domain of PKC-delta is a phosphotyrosine binding domain. *Cell* 2005; **121**: 271–80.
8 Ikeda JI, Morii E, Kimura H *et al*. Epigenetic regulation of the expression of the novel stem cell marker CDCP1 in cancer cells. *J Pathol* 2006; **210**: 75–84.
9 Kimura H, Morii E, Ikeda JI *et al*. Role of DNA methylation for expression of novel stem cell marker CDCP1 in hematopoietic cells. *Leukemia* 2006; **20**: 1551–6.
10 Uekita T, Jia L, Narisawa-Saito M, Yokota J, Kiyono T, Sakai R. CUB domain-containing protein 1 is a novel regulator of anoikis resistance in lung adenocarcinoma. *Mol Cell Biol* 2007; **27**: 7649–60.
11 Sobin LH, Wittekind Ch, eds. *TNM Classification of Malignant Tumors*, 5th edn. New York: John Wiley & Sons, Inc., 1997.
12 Uekita T, Tanaka M, Takigahira M *et al*. CUB domain-containing protein 1 regulates peritoneal dissemination of gastric scirrhous carcinoma. *Am J Patho* 2008; **172**: 1729–39.
13 Suzuki K, Nagai K, Yoshida J *et al*. Conventional Clinicopathologic Prognostic Factors in Surgically Resected Nonsmall Cell Lung Carcinoma. *Cancer* 1999; **86**: 1976–84.