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Protein interacting with C α kinase 1 (PICK1) is involved in promoting tumor growth and correlates with poor prognosis of human breast cancer

Bin Zhang,[1,2,7] Wenfeng Cao,[3,7] Fei Zhang,[4] Lin Zhang,[4] Ruifang Niu,[4] Yun Niu,[1,5] Li Fu,[1,5] Xishan Hao[1] and Xuchen Cao[1,2,6]

[1]National Key Laboratory of Breast Cancer Prevention and Treatment, [2]Department of Breast Cancer Surgery of the Cancer Hospital, [3]Department of Pathology of the Cancer Institute, [4]Tianjin Key Laboratory of Cancer Prevention and Treatment, [5]Department of Breast Cancer Pathology of the Cancer Hospital, Tianjin Medical University, Tianjin, China

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Protein interacting with C α kinase 1 (PICK1), which interacts with multiple different proteins in a variety of cellular contexts, is believed to play important roles in diverse pathological conditions including cancer. In this study, we attempted to investigate the correlation of PICK1 with clinicopathological features as well as prognosis of human breast cancer. In addition, we aimed at a better understanding of the biological function of PICK1 in breast cancer cell biology. As judged by semi- quantitative RT-PCR and western blotting, PICK1 was overexpressed in tumor cells as compared to adjacent normal epithelia in breast, lung, gastric, colorectal, and ovarian cancer. As judged by immunostaining breast cancer tissue microarrays, high levels of PICK1 expression correlated with shortened span of overall survival (OS). Protein interacting with C α kinase 1 (PICK1) expression seemed to be specifically associated with reduced OS in lymph node-positive, Her/neu-2 positive, and the basal-like type subgroups, respectively. Consistently, the expression of PICK1 correlated with histological grade, lymph node metastasis, Her-2/neu-positivity, and triple-negative basal-like breast cancer. Protein interacting with C α kinase 1 (PICK1) was not correlated with menopausal status, tumor size, or hormone receptor status. In a complementary study, transfection of MDA-MB-231 cells with PICK1 siRNA decreased cell proliferation and colony formation *in vitro* and inhibited tumorigenicity in nude mice. Our clinical and experimental evidence supports an oncogenic role of PICK1 in human breast cancer. In particular, our data suggest that PICK1 promotes tumor cell proliferation. Taken together, PICK1 may serve not only as a marker for poor prognosis, but also as a therapeutic target in breast cancer. (*Cancer Sci* 2010; 101: 1536–1542)

Breast cancer is one of the leading causes of cancer death among women worldwide.[1] The incidence and mortality rate of breast cancer have increased sharply in China over the past few decades,[2] whereas the breast cancer mortality rate in developed countries has declined in the same period, likely due to the implementation of screening,[3] improvements in early breast cancer management,[4] and improved systemic adjuvant treatments.[5] Currently, the strategy for breast cancer management is primarily based on the traditional prognostic and predictive factors including histologic, clinical, and some well-defined molecular profiles. Since the biomarker profile may provide valuable insights into the underlying mechanisms of disease progression,[6,7] in light of the well-documented racial disparities in breast cancer incidence and prognosis, it is important to identify and validate specific biomarkers for Chinese breast cancer patients.

Protein interacting with C α kinase 1 (PICK1), a PSD95/disk-large/ZO-1 (PDZ) domain-containing protein, was originally identified by a yeast two-hybrid system on the basis of its interaction with protein kinase C α (PKCα).[8,9] In addition to PKCα,PICK1 also interacts with glutamate receptors (GluRs),[10–13] prolactin-releasing peptide (PrRP),[14] acid-sensing ion channel (ASICs),[15–17] dopamine transporter (DAT),[18–21] Karlirin 7,[22] ephrin type-B receptor 2 (EphB2),[20] ErbB2/Her-2,[23] TIS21,[24] coxsackie virus and adenovirus receptor (CXADR),[25] and UNC5H.[26] By serving as an adaptor of these proteins as well as an organizer of their subcellular localization,[26] PICK1 is located in the cytoplasm of cancer cells and believed to play an important role in cancer,[23–25] schizophrenia,[18,19] pain,[15,17] and Parkinson's disease.[20,21] Interestingly, the relevance of the PICK1 expression profile in cancer has not been rigorously evaluated. The aim of this study was to analyze PICK1 expression in normal and cancerous breast tissues, and to investigate the biological function of PICK1 in experimental models of breast cancer.

Materials and Methods

Human subjects and tissue specimens. A series of fresh resection specimens from 114 cases of breast cancer, 104 cases of lung cancer, 41 cases of gastric cancer, 30 cases of colorectal cancer, and 31 cases of ovarian cancer were taken from cancer patients between June 2003 and December 2007 at the Department of Breast Cancer Surgery, Tianjin Medical University Cancer Institute and Hospital. The Institutional Review Board at the National Key Laboratory of Breast Cancer Prevention and Treatment approved the study. Tumor tissues and matching normal tissues that were harvested from the resection margin (before the storage) were dissected and stored at −70°C.

A total of 496 retrospective paraffin-embedded breast tissue specimens were harvested from patients with invasive breast cancer at the Department of Breast Cancer Surgery, Tianjin Medical University Cancer Institute and Hospital. Patients had not received radiotherapy or neoadjuvant therapy prior to the surgery. After the surgery, all patients received six courses of cyclophosphamide/methotrexate/fluorouracil adjuvant chemotherapy. In addition, patients with lymph node metastasis or tumor size ≥5 cm received radiation. The patients with ER^+/PR^+ tumors were treated for 5 years with tamoxifen.

The median age of the patients was 50.3 years. Among the 496 cases, 250 women were premenopausal and 246 were postmenopausal. At the time of operation, 128 cases (25.8 %) were grade I tumors, 224 (45.2%) cases were grade II, and 144 cases (29.0%) were grade III. Tumor size was ≤2 cm in 86 cases

[6]To whom correspondence should be addressed. E-mail: eeflying@163.com
[7]Contributed equally to this work.

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(17.3%), whereas 410 cases (82.7%) had tumors >2 cm. The clinical stage was assessed by the surgical pathologists according to the 2003 TNM classification criteria by the International Union Against Cancer. A total of 242 cases (48.8%) were lymph node negative, 107 cases (21.6%) were N1, 50 cases (10.0%) were N2, and 97 cases (19.6%) were N3. All patients, unless deceased, were followed up for at least 36 months and up to 173 months or until they deceased. The outcome was defined by the months of post-surgery overall survival (OS). Consent was obtained from each patient after full explanation of the purpose of the study and nature of all procedures used.

Cell culture. The breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection (Manassas, VA, USA), and was cultured in RPMI-1640 medium supplemented with 10% FCS in a humidified incubator containing 5% CO_2 at 37°C.

Tissue microarrays (TMA) and immunohistochemistry (IHC). Construction of TMA and detection of IHC were both performed using a method as described previously.[27] For detecting PICK1, estrogen receptor (ER), progesterone receptor (PR), and Her-2/neu, antigen retrieval was modified to include 5-min high-power microwave followed by 10-min low-power microwave in phosphate-buffered saline (PBS; pH 7.0). The antibodies and the dilution factors were as follows: polyclonal antibody against PICK1 (1:75) from ProteinTech Group (Chicago, IL, USA); Dako Clone ID5 antibody against ER (1:450) (Carpinteria, CA, USA); Dako Clone IA6 antibody against PR (1:200); and Dako polyclonal antibody against Her-2/neu (1:1000). Breast cancer cell line MDA-MB-231 known to express a high level of PICK1 was used as a positive control for these proteins. Breast cancer cell lines MCF-7 and T47D were used as positive controls for ER and PR. Ovarian cancer cell line SK-OV-3 was used as a positive control for Her-2/neu staining.[28] Preimmune serum was used in the place of the primary antibodies in all negative controls of IHC.

Immunohistochemical (IHC) quantification. Immunohistochemistry (IHC) of PICK1 was evaluated independently by two pathologists who were both blinded to patients' clinicopathologic parameters and outcomes. Discordant scores were re-evaluated by the investigators and the consensus scores were used for further analyses. In order to score, we counted five high-power fields (×400) of microscopy, and for one high-power field, we counted 50 cells. Both the intensity and extent of IHC were assessed.[27] The intensity of the immunostaining was defined by the negative and positive controls according to four categories: 0 for no staining; 1 for light staining; 2 for moderate staining; and 3 for dark staining. The percentage of positive cells, as the extent of immunostaining, was quantified under microscope and classified into four groups: 1 for <25% positive cells; 2 for 25–50% positive cells; 3 for 51–75% positive cells; and 4 for >75% positive cells. The staining index (SI), the product of the intensity and the percentage of positive staining, was used to define high (SI ≥6) or low (SI <6) expression. The criterion of the Herceptest/Pathway system[29] was followed to score Her-2/neu IHC. The criteria for scoring of ER and PR were similar.[27]

Semi-quantitative RT-PCR. Total RNA was extracted from frozen tissues using TRIzol reagent according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA, USA). The quality of the RNA was assessed by 1% denaturing agarose gel electrophoresis and spectrophotometry. One microgram of total RNA from each sample was reverse-transcribed using the Transcriptase SuperScript II Preamplification System for First Strand cDNA kit (Invitrogen). The primers for detecting PICK1 cDNA were: 5′-TAAGGTGGAGGTGGCGAAGATGATT-3′ (sense), and 5′-AGCCGGTGCTTGACTTTCTTCAACA-3′ (antisense). The primers for detecting internal control β-actin were: 5′-CAG AGC AAG AGA GGC ATC C-3′ (sense) and 5′-CTG GGG TGT TGA AGG TCT C-3′ (antisense). After an initial denaturation at 95°C for 2 min, cycling conditions were as follows: 35 cycles: 95°C for 10 s, 60°C for 20 s, and 72°C for 20 s.

Western blotting. Cells or tissues were lysed with the lysis buffer (1% SDS, 10 mmol/L Tris–Cl [pH 7.6], 20 μg/μL aprotinin, 20 μg/μL leupeptin and 1 mmol/L 4-[2-aminoethyl] benzenosulfonyl fluoride). Supernatants were collected and analyzed for protein concentrations using the BCA Protein Assay kit (Pierce, Rockford, IL, USA). Ten micrograms of protein extract from each sample were separated on a 12% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. After blocking, the membrane was incubated with 1000-fold diluted anti-PICK1 antibody (ProteinTech Group) at 4°C overnight. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:3000 at room temperature for 1 h. The bound secondary antibody was detected with the ECL kit (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA). For the loading control, western membrane was stripped and reprobed by anti-β-actin antibody (Sigma, St. Louis, MO, USA) at the dilution recommended by the manufacturer.

Protein interacting with C α kinase 1 (PICK1) siRNA construct and transfection. To construct a vector for PICK1 siRNA, the pRNA-U6.1/Neo (GenScript, Piscataway, NJ, USA) was digested with BamH I and HindIII (Takara Bio, Shiga, Japan). Chemically synthesized oligonucleotides encoding PICK1-targeting small-interfering RNA and an appropriate loop motif were inserted downstream of the U1 promoter using a DNA ligation kit (Takara Bio) and subcloned. The sequences of PICK1-interference oligonucleotide produced by these constructs were: 5′-GTTCGGCATTCGGCTTCTG-3′, corresponding to positions 865–883 within the PICK1 mRNA sequence (38); and 5′-GATGATTCAGGAGGTGAAG-3′, corresponding to positions 472–490 within the PICK1 mRNA. The choices of the siRNA sequences were made based on an analysis using Oligo 6.0 software (National Biosciences, Plymouth, MN, USA). Furthermore, each siRNA sequence appeared to be specific to PICK1 based on a BLAST search. For the negative control, a pRNA-U6.1/Neo containing a scrambled sequence was used. MDA-MB-231 cells were transfected with either the siRNA construct or the empty vector using Lipofectamine 2000 (Invitrogen). After G418 (400 μg/μL; Life Technologies, Rockville, MD, USA) screening, stable clones were identified by semi-quantitative RT-PCR and western blot analysis.

Cell growth kinetics. MDA-MB-231 cells, transfected with either PICK1 siRNA-expressing vector or the empty vector, were seeded in 96-well plates at the density of 1×10^3/well. MTT dissolved in RPMI-1640 at the final concentration of 0.5 mg/μL was added to cells that were incubated for 1, 2, 3, 4, 5, 6, and 7 days, respectively. Four hours later, 200 μL of DMSO was added to each well to solubilize the formazan crystals. Spectrometric absorbance at 570 nm was measured using a Bio-Kinetics Reader (Bio-Rad, Hercules, CA, USA).

Clonogenicity assay. MDA-MB-231 cells, transfected with either PICK1 siRNA-expressing or empty vector, were seeded in six-well plates at the density of 5×10^2/well. The plates were incubated until colonies became visible (2–4 weeks). Cells were fixed with methanol, and stained with Giemsa. The number of colonies was counted under a microscope.

Cell proliferation assay by flow cytometry. Empty vector and PICK1 siRNA-transfected MDA-MB-231 cells were grown to 80–90% confluence, harvested, washed twice with PBS, and fixed overnight at 4°C in 70% ethanol. After washing twice with PBS, cells were incubated for 1 h at room temperature in a PBS-based solution containing 5 μg/μL propidium iodide and 50 μg/μL RNase A. Flow-activated cell sorter analysis was carried out using a FACS Calibur flow cytometer (Becton

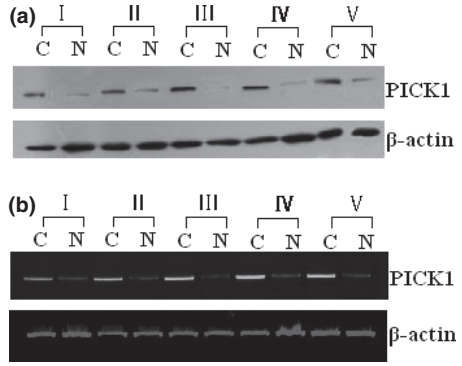

Fig. 1. Western blotting (a) and semi-quantitative RT-PCR (b) of protein interacting with C α kinase 1 (PICK1) in patient-matched cancerous and normal tissues. Representative RT-PCR and western blots are shown. (I) Breast cancer; (II) lung cancer; (III) gastric cancer; (IV) colorectal cancer; and (V) ovarian cancer. As an internal control of western blotting, the same membrane was re-probed with β-actin antibody. C, cancerous tissue; N, patient-matched normal tissue.

Dickinson, Mountain View, CA, USA) equipped with CellQuest software. A total of 10 000 events were measured per sample.

Xenograft tumor growth in nude mice. Each testing cell line was harvested from culture dishes and made into a single-cell suspension in the maintenance media (without G418). The cell viability was >95% as determined by Trypan blue staining. Cells (2×10^6) in 0.1 mL of PBS were inoculated into the mammary fatpad of 8-week-old female SCID BALB/c mice (five for each group). At the end of 8 weeks, all mice were sacrificed and the tumors were excised and weighed.

Statistical analyses. Statistical analyses were performed using SPSS statistical software (SPSS, Chicago, IL, USA). The correlation between PICK1 expression and clinicopathological features, prospective outcomes characteristics, was analyzed using Spearman's correlation analysis. Patients' OS data were further stratified by lymph node status, Her-2/neu status, hormone receptor status, and additional molecular markers. Unless otherwise specified, a χ^2-test was performed. Data from MTT assay, clonogenicity assay, and tumorigenicity assay were presented as means ± SD. In all statistic analyses, *P*-values of <0.05 were considered statistically significant.

Results

Overexpression of PICK1 in human tumor tissues. We measured PICK1 expression in matched tumorous and normal tissues from human breast cancer, lung cancer, gastric cancer, colorectal cancer, and ovarian cancer specimens by semi-quantitative RT-PCR. As shown in Figure 1(a), overexpression of PICK1 was observed in cancer tissues as compared to the matching normal tissues in all tumor specimens (Table 1). Similar results were obtained from western blotting with the antibody against PICK1. Based on the consensus of PCR data and western blots, PICK1 was found to be up-regulated in 41.23% of breast cancer samples (47/114, $P < 0.001$), 44.23% of lung cancer samples (46/104, $P < 0.001$), 51.21% of gastric cancer samples (21/41, $P < 0.001$), 36.67% of colorectal cancer samples (11/30, $P = 0.016$), and 19.34% of ovarian cancer samples (6/31, $P = 0.005$) compared with their normal counterparts (Fig. 1b, Table 1).

Correlation of PICK1 with lymph node infiltration, tumor grade, and her-2/neu status in human breast cancer. Immunohistochemistry (IHC) of PICK1 in TMA constructed with archival tissue blocks of breast cancer showed that strong positive staining, which was mainly located in the cytoplasm of cancerous cells (Fig. 2), correlated with lymph node metastasis ($P < 0.0001$), higher tumor grade ($P < 0.0001$), and Her-2/neu positive ($P < 0.0001$), but not with tumor size (Table 2). Pearson χ^2 analysis was performed and *P*-values of <0.05 were considered statistically significant. A significant correlation between PICK1 and Her-2/neu staining in cancer cells ($P = 0.019$) was observed. Protein interacting with C α kinase 1 (PICK1) did not appear to be regulated by sexual steroid hormones, since it was correlated neither with the menopausal statuses of the patients, nor with ER or PR status.

Protein interacting with C α kinase 1 (PICK1) and poor prognosis of invasive breast carcinoma. Based on the log-rank analyses, high level of PICK1 expression in cancer cells correlated with decreased OS ($P < 0.0001$, Fig. 3a). When patients were further stratified by lymph node status, Her-2/neu status, hormone receptors status, and molecular subclassification, PICK1 expression in cancer cells was found to specifically correlate with lymph node-positive subgroups ($P < 0.0001$, Fig. 3b). For molecular subclassification, PICK1 expression was associated with reduced OS in both ER/PR$^-$HER-2$^+$ (Fig. 3g) and ER/PR$^-$HER-2$^-$ (Fig. 3f) ($P = 0.041$ and $P = 0.002$, respectively), but not in ER/PR$^+$HER-2$^-$ (Fig. 3e) or ER/PR$^+$HER-2$^+$ (Fig. 3h) cases (Table 3). In Cox regression models, univariate survival analyses showed that tumor size, lymph node positivity, histological grades, hormone receptor, Her-2/neu, and PICK1 overexpression were each associated with a worse prognosis, while multivariate survival analyses showed that tumor size, lymph node positivity, histological grades, Her-2/neu, and PICK1 overexpression by cancer cells were independent adverse prognostic factors for overall survival (Table 4). Parallel analyses showed that hormone receptors status was not correlated with OS.

Effect of PICK1 silencing on tumor cell proliferation *in vitro*. Semi-quantitative RT-PCR and western blotting showed that MDA-MB-231 cells expressed abundant endogenous PICK1 (Fig. 4a). To define whether PICK1 promotes the growth of breast cancer, MDA-MB-231 cells were chosen for PICK1 siRNA expression. After G418 screening, two clones that expressed PICK1 siRNA (231-Si-3 and 231-Si-6) and one clone that express the empty vector (231-Si-vector) were identified (Fig. 4b). There was no significant difference in growth rates between MDA-MB-231 and empty vector cells. However, MTT assay and clonogenicity assay showed that cell viability and proliferation of 231-Si-3

Table 1. Overexpression of PICK1 in human breast, lung, gastric, colorectal, and ovarian cancers

	Semi-quantitative RT-PCR				*P*-value	Western blotting				*P*-value
	Cancer		Normal			Cancer		Normal		
Breast cancer	39/114	34.21%	6/114	5.26%	<0.001	47/114	41.23%	9/114	7.89%	<0.001
Lung cancer	40/101	39.60%	7/101	6.93%	<0.001	46/104	44.23%	10/104	9.62%	<0.001
Gastric cancer	17/39	43.58%	3/39	7.69%	<0.001	21/41	51.21%	5/41	12.20%	<0.001
Colorectal cancer	9/30	30.0%	4/30	13.33%	0.023	11/30	36.67%	5/30	16.67%	0.016
Ovarian cancer	5/31	16.13%	3/31	9.67%	0.017	6/31	19.35%	2/31	6.45%	0.005

PICK1, protein interacting with C α kinase 1.

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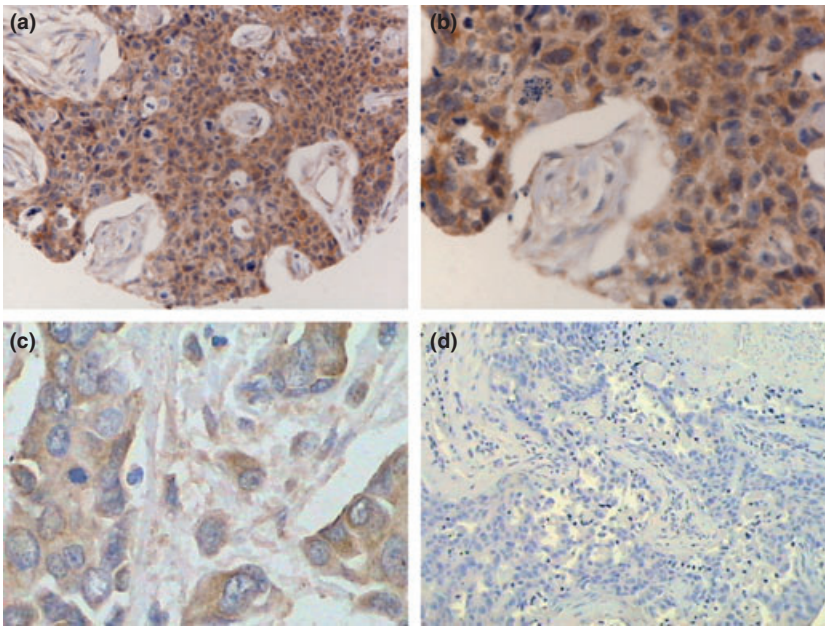

Fig. 2. Representative immunohistochemical staining of protein interacting with C α kinase 1 (PICK1). Note that the specific staining of PICK1 in breast cancer was primarily located in the cytoplasm of cancerous cells. (a) PICK strong positive staining (×200); (b) strong positive staining (×400); (c) PICK weak positive staining (×400); (d) negative staining (×200).

Table 2. Correlation of PICK1 expression with clinicopathological parameters and other biomarkers

Parameters/Markers	Total	High PICK1 expression				*P*-value
		+		–		
		n	%	*n*	%	
Menopausal						
Pre-menopausal	250	69	27.6	181	72.4	0.847
Post-menopausal	246	66	26.8	180	73.2	
Tumor size						
<2 cm	86	23	26.7	63	72.3	0.914
>2 cm	410	112	27.3	298	72.7	
Lymph node status						
N0	242	39	16.1	203	83.9	**<0.0001**
N1	107	19	17.8	88	82.2	
N2	50	17	34.0	33	66.0	
N3	97	60	61.9	37	38.1	
Histological grade						
G1	128	21	16.4	107	83.6	**<0.0001**
G2	224	64	28.6	160	71.4	
G3	144	50	34.7	94	65.3	
ER status						
negative	196	60	30.6	136	69.4	0.170
positive	300	75	25.0	225	75.0	
PR status						
negative	304	89	29.3	215	70.7	0.195
positive	192	46	24.0	146	76.0	
HER2/neu status						
negative	399	93	23.3	306	76.7	**<0.0001**
positive	97	42	43.3	55	56.7	
HR&HER status						
HR^+HER^+	54	23	42.6	31	57.4	**<0.0001**
HR^+HER^-	288	64	22.2	224	77.8	
HR^-HER^+	43	19	44.2	24	55.8	
HR^-HER^-	111	29	26.1	82	73.9	

ER, estrogen receptor; HR, hormone receptor; HER, Her-2/neu; PICK1, protein interacting with C α kinase 1; PR, progesterone receptor. Statistical significance if the *P*-value is less than 0.05 ($P < 0.05$).

and 231-Si-6 were inhibited compared to that of parental MDA-MB-231 cells or 231-Si-vector cells. Flow cytometry assay was performed to determine whether inhibition of cell growth and proliferation reflected cell cycle arrest at any particular phases. As shown in Figure 4, PICK1 siRNA expression resulted in a cell cycle arrest in the S phase by 13.4–14.5%, when compared to empty vector-transfected control cells. No significant apoptosis of PICK1 siRNA-transfected MDA-MB-231 cells was observed under the experimental condition.

Effects of PICK1 silencing on xenograft tumor growth in nude mice. Four groups of nude mice were inoculated with 231-Si-3, 231-Si-6, 231-Si-vector, or parental MDA-MB-231 cells, respectively. As shown in Figure 4, PICK1 siRNA-transfected cells grew at a significantly slower rate than parental MDA-MB-231 or 231-Si-vector cells. At the time of sacrificing the animals, 8 weeks after tumor inoculation, the weights of the tumors derived from 231-Si-3 or 231-Si-6 cells were significantly less than those of the tumors derived from parental MDA-MB-231 cells or 231-Si-vector cells.

Discussion

Predicting the prognosis is clearly one the most challenging issues in cancer management.[30–32] In breast cancer, racial, geographical, and dietary factors have all been considered for their impacts on cancer incidence and patient survival.[33] At the histopathological level, breast cancer is a highly heterogeneous disease, which hampers clear prognosis predictions.[34,35] Tumor size, node status, historical grade, hormone receptors (HR), and Her-2/neu status have been used with limited success as the biomarkers.[36–39] Currently, breast cancer is commonly subclassified on the basis of the presence of ER, PR, and the HER-2/neu status. Breast cancers can be divided into $HR^+/HER\text{-}2^-$, $HR^+/HER\text{-}2^+$, $HR^-/HER\text{-}2^+$, and $HR^-/HER\text{-}2^-$ (triple-negative).[40–42] This classification has proven useful in terms of predicting prognosis and guiding treatment strategies. For example, triple-negative breast cancers are insensitive to most available hormonal therapeutic agents, and are frequently resistant to standard chemotherapeutic regimens.[41,43] This subgroup accounts for 15% of all types of breast cancer and for a higher

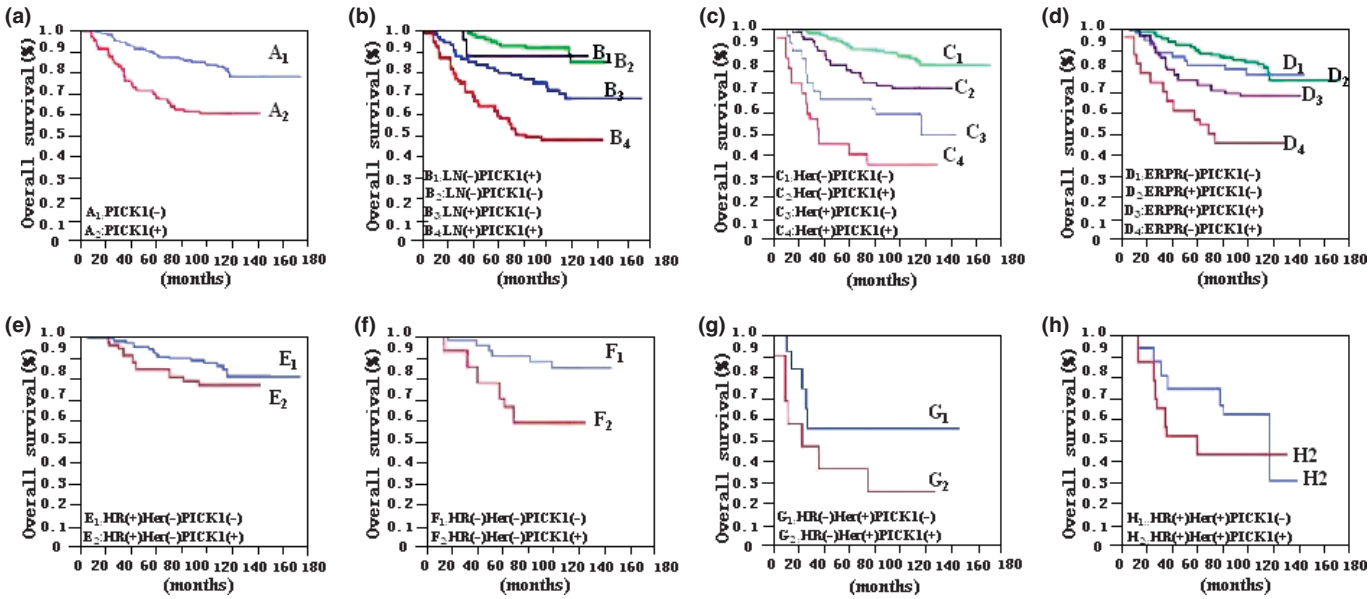

Fig. 3. The prognostic significance of high protein interacting with C α kinase 1 (PICK1) protein expression in the entire patient population. The graphs show the effect of PICK1 expression within cancer cells (a) on patient overall survival (log-rank test) and stratified analysis by lymph nodes status (b), Her-2/neu status (c), hormone receptor (HR) status (d), and molecular subclassification (e–h). High PICK1 expression in cancer cells (a) associated with decreased overall survival (OS) ($P < 0.0001$, respectively). When patients' OS were stratified by lymph node status, Her-2/neu status, hormone receptor status, and molecular subclassification status, PICK1 expression was associated with reduced OS in the Her-2/neu-positive and -negative (c, $P = 0.019$ and $P = 0.003$, respectively), HR-positive and -negative (d, $P = 0.007$ and $P < 0.0001$, respectively), and lymph node -positive subgroups (b, $P < 0.0001$). Molecular subclassification: $HR^-/HER\text{-}2^+$ and $HR^-/HER\text{-}2^-$ (g–f, $P = 0.0.041$ and $P = 0.002$, respectively).

Table 3. Correlation of PICK1 expression with OS of overall and stratified subpopulations

	PICK1	*n*	E	5 ys%	10 ys%	OS (95% CI)	*P*-value
Overall							
	Low	361	68	87.6	77.3	148.0 (142.5–153.4)	**<0.0001**
	High	135	52	67.5	60.2	100.3 (91.2–109.4)	
Lymph node status							
Negative	Low	203	22	93	84.4	134.6 (130.5–138.7)	0.818
	High	39	5	87.2	87.2	118.7 (108.6–128.8)	
Positive	Low	158	46	80.5	67.3	134.1 (124.5–143.7)	**<0.0001**
	High	96	47	59.2	48.2	88.1 (76.9–99.4)	
Her-2/neu status							
Negative	Low	306	45	91.3	82.4	154.6 (149.6–159.6)	**0.003**
	High	93	25	80.0	71.5	114.9 (105.7–124.2)	
Positive	Low	55	23	66.3	49.6	96.8 (81.6–112.0)	**0.019**
	High	42	27	40.5	35.7	64.3 (48.6–80.1)	
Hormone receptor status							
Negative	Low	106	22	82.8	78.4	122.7 (114.1–131.2)	**<0.0001**
	High	48	25	57	46.1	77.5 (62.9–92.1)	
Positive	Low	255	46	89.6	75.7	148.7 (142.2–155.2)	**0.007**
	High	87	27	73.3	68.0	109.5 (99.2–119.9)	
HR&HER status							
HR^+HER^+	Low	31	13	74.2	31.2	97.4 (79.6–115.3)	0.192
	High	23	13	43.5	43.5	74.5 (53.7–95.2)	
HR^+HER^-	Low	224	33	91.7	81	154.0 (147.9–160.1)	0.152
	High	64	14	84.3	76.9	120.5 (110.4–130.7)	
HR^-HER^+	Low	24	10	55.6	55.6	89.8 (64.1–115.5)	**0.041**
	High	19	14	36.8	26.3	50.7 (28.7–72.8)	
HR^-HER^-	Low	82	12	90.2	84.7	131.8 (124.6–139.0)	**0.002**
	High	29	11	70.4	59.3	94.7 (78.5–111.0)	

CI, confidence interval; E, events of total mortality; HER, Her-2/neu; HR, hormone receptor; *n*, number of patients; OS, overall survival in months; PICK1, protein interacting with C α kinase 1; ys, years of survival. Statistical significance if the *P*-value is less than 0.05 ($P < 0.05$).

percentage of breast cancer arising in African and African-American women who are premenopausal.[43] Interestingly, however, Chinese women in our series have a higher proportion of triple-negative breast cancer (26.1%).

Research efforts aimed at systematically identifying the profiles of differential gene and protein expression in Chinese women are critically needed. The present study provides the first evidence that PICK1 may be used as a novel independent

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Table 4. Univariate and multivariate Cox survival analysis

Variables	*P*-values	Hazard rate	(95% CI)
Univariate			
Menopausal status	0.112	0.896	(0.565–1.420)
Tumor size	**0.024**	1.312	(1.058–1.628)
Lymph node status	**<0.001**	2.214	(1.338–3.664)
Histological grade	**0.038**	1.389	(1.019–1.893)
Hormone receptor	**0.041**	0.534	(0.315–0.905)
Her-2/neu	**<0.001**	2.012	(1.400–2.891)
High PICK1	**0.041**	1.268	(1.010–1.592)
Multivariate			
Histological grade	**0.034**	1.393	(1.034–1.876)
Lymph node status	**<0.001**	2.219	(1.359–3.622)
Her-2/neu	**<0.001**	2.019	(1.416–2.879)
Tumor size	**0.019**	1.318	(1.105–1.572)
High PICK1	**0.038**	1.273	(1.018–1.592)

PICK1, protein interacting with C α kinase 1. Statistical significance if the *P*-value is less than 0.05 ($P < 0.05$).

biomarker for breast cancer prognosis. We have also detected overexpression of PICK1 in many other types of human cancer; whether PICK1 may help predict a poor prognosis in those cancers needs further study. The multivariate statistic analyses indicate that besides lymph node status, and Her-2/neu and molecular subclassification status expression, PICK1 may be particularly useful as a prognostic marker in human invasive breast carcinoma, especially when evaluated along with triple-negative patients ($P = 0.002$).

In our study, high levels of PICK1 in cancer cells correlated with the expression of the Her-2/neu protein. Currently, it is not known whether PICK1 is regulated by Her-2/neu, but some evidence indicates a cross-talk between PKCα, PICK1 ligand proteins, and Her-2/neu molecules.[44] Jaulin-Bastard *et al.* showed that PICK1 is involved in the localization of ErbB2 at specific sites in polarized cells and participates in the formation of protein complexes associated with receptors with tyrosine kinases activity.[23] That said, it is important to note that PICK1 seems to be of prognostic value even for the Her-2/neu-positive subset of cases, suggesting a Her-2/neu-independent function of PICK1 in promoting breast cancer progression.

Consistent with the clinical finding that PICK1 correlates with high tumor grades, lymph node infiltration, and poor prognosis of breast cancer, we tested the hypothesis that PICK1 may act as a tumor-promoting factor. We showed that silencing PICK1 expression in MDA-MB-231 breast cancer cells led to significant inhibition of cell proliferation and cell cycle arrest in the S phase *in vitro* and inhibited xenograft tumor growth in nude mice. Further studies are underway to elucidate the mechanisms underlying the antiproliferative effects of PICK1 in breast cancer cells.

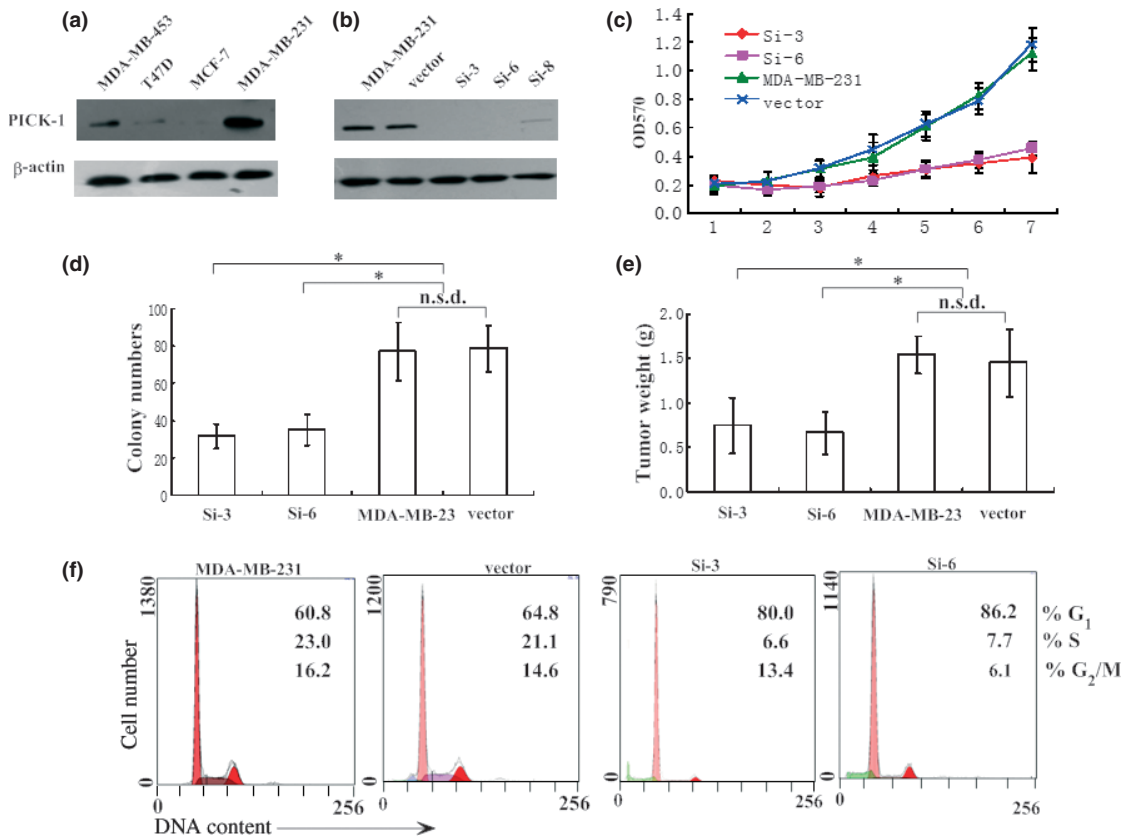

Fig. 4. Expression of protein interacting with C α kinase 1 (PICK1) in breast cancer cell lines and the effects of PICK1 on tumor growth and tumorigenicity. (a) Breast cancer cell lines with endogenous PICK1 expression were screened by semi-quantitative RT-PCR and western blotting. (b) Positive clones transfected with siRNA were identified by semi-quantitative RT-PCR and western blotting. Si-3 and Si-6 were different individual transfectants. Si-3 and Si-6 showed obviously reduced PICK1 expression and were chosen for further analysis. (c) Growth curve by MTT assay; siRNA transfectants Si-3 and Si-6 showed obviously reduced cell growth compared with controls (vector and MDA-MB-231). (d) Clonogenicity assay showing that silence transfectants Si-3 and Si-6 had lower clonogenicity as compared with controls (vector and MDA-MB-231). (e) Tumorigenicity of clones Si-3 and Si-6 compared with controls (vector and MDA-MB-231). Tumor weight from MDA-MB-231 cells, empty vector transfectants, and cell clone Si-3 and Si-6 cells are shown as mean ± SD of three independent experiments. (f) Flow cytometry analysis revealed that Si-3 and Si-6 caused S-phase arrest compared with controls (vector and MDA-MB-231). *$P < 0.001$; n.s.d., no significant difference, Si-3 and Si-6, clone 3, and clone 6 of PICK1 siRNA-transfected MDA-MB-231; vector, MDA-MB-231-transfected with the empty vector; MDA-MB-231, parental cells.

In conclusion, our data suggest that PICK1 may serve as a novel independent prognostic marker in invasive breast cancer patients. Moreover, PICK1 may be a potential therapeutic target of breast cancer.

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Disclosure Statement

The authors have no conflict of interest.

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