PDZ-Binding Kinase/T-LAK Cell-Originated Protein Kinase, a Putative Cancer/Testis Antigen with an Oncogenic Activity in Breast Cancer

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Abstract
Breast cancer is one of the most common cancers among women. To discover molecular targets that are applicable for development of novel breast cancer therapy, we previously did genome-wide expression profile analysis of 81 breast cancers and found dozens of genes that were highly and commonly up-regulated in breast cancer cells. Among them, we here focused on one gene that encodes PDZ-binding kinase/T-LAK cell-originated protein kinase (PBK/TOPK), including a kinase domain. Northern blot analyses using mRNAs of normal human organs, breast cancer tissues, and cancer cell lines indicated this molecule to be a novel cancer/testis antigen. Reduction of PBK/TOPK expression by small interfering RNA resulted in significant suppression of cell growth probably due to dysfunction in the cytokinetic process. Immunocytochemical analysis with anti-PBK/TOPK antibody implicated a critical role of PBK/TOPK in an early step of mitosis. PBK/TOPK could phosphorylate histone H3 at Ser\(^{10}\) in vitro and in vivo, and mediated its growth-promoting effect through histone H3 modification. Because PBK/TOPK is the cancer/testis antigen and its kinase function is likely to be related to its oncogenic activity, we suggest PBK/TOPK to be a promising molecular target for breast cancer therapy. (Cancer Res 2006; 66(18): 9186-95)

Introduction
Breast cancer is one of the most common cancers worldwide and has been significantly increasing (1). To reduce the mortality rate and to make the quality of life to the patients better, various approaches, including early detection with mammography and development of molecular-targeted therapeutic drugs, such as tamoxifen and trastuzumab, have been taken. Due to such efforts, the mortality rates in Western societies have been improved, but at present the very limited treatment options can be available to patients at an advanced stage, particularly those with hormone-independent type. Hence, novel molecular-targeted drugs to provide better management to such patients are still eagerly expected.

cDNA microarray has been used as effective tools to analyze expression patterns of thousands of genes simultaneously. Comparison of genome-wide expression profiles between cancers and normal cells by cDNA microarray provides useful information to discover candidate target molecules for development of diagnosis and treatment of cancer (2). Through genome-wide expression analysis, we have isolated a number of oncogenes that were involved in development and/or progression of hepatocellular carcinomas (3, 4), synovial sarcomas (5, 6), and renal cell carcinomas (7). Such molecules are considered to be good candidate molecules for development of new therapeutic modalities.

Because cytotoxic drugs often cause severe adverse reactions, it is obvious that thoughtful selection of novel target molecules on the basis of well-characterized mechanisms of action should be very helpful to develop effective anticancer drugs with the minimum risk of side effects. Toward this goal, we previously did the expression profile analysis of 81 breast cancers (8) and 29 normal human tissues (9). Among the genes up-regulated in breast cancers, we focused on PDZ-binding kinase/T-LAK cell-originated protein kinase (PBK/TOPK), which is significantly overexpressed in the great majority of breast cancer cases examined. PBK/TOPK was first identified as aDlg1-interacting protein by yeast two-hybrid screening and characterized as a mitotic kinase with PDZ-binding motif at COOH terminus (10). PBK/TOPK was also identified by the other group as a mitogen-activated protein kinase (MAPK) kinase (MAPKK)–like protein kinase (11).

Cancer/testis antigens can be defined by predominant expression in various types of cancer and undetectable expression in normal tissues except germ cells in testis. To date, 44 distinct cancer/testis antigen families have been identified with particular attention as potential targets for tumor immunotherapy (12). It has been reported that various cancer/testis antigens were expressed relatively commonly in breast cancers, suggesting their potentials for development of targeted therapy for breast cancer (13). Indeed, some of cancer/testis antigens, like NY-ESO-1 and NY-RR-1, are applied as cancer vaccines in early clinical trials for breast cancer patients (14).

Here, we report overexpression of PBK/TOPK in the great majority of breast cancer cells and that its kinase activity is likely to play an important role in mammary carcinogenesis. Furthermore, the fact that PBK/TOPK expression pattern as the cancer/testis antigen suggests PBK/TOPK to be a promising molecular target for breast cancer therapy through cancer vaccine–mediated immunotherapy and/or inhibition of PBK/TOPK–specific kinase function.

Materials and Methods
Breast cancer cell lines and clinical samples. Human breast cancer cell lines BT-20, HCC1937, MCF-7, MDA-MB-435 S, SKBR3, T47D, and YMB1, as well as immortalized human mammary cell-line HBL100, are purchased from American Type Culture Collection (Rockville, MD), and cultured under the recommendations of their respective depositors. Human mammalian epithelial cell (HMEC) is purchased from Cambrex Bio Science (Walkersville, MD). HBC4, HBC5, and MDA-MB-231 cell lines are kind gifts from

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PBK/TOPK as a Novel Molecular Target for Breast Cancer

Dr. Yamori (Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan). All cells were cultured in an appropriate medium, i.e., RPMI 1640 (Sigma-Aldrich, St. Louis, MO) for HBC4, HBC5, HCC1937, T47D, and YMB1 (with 2 mmol/L L-glutamine); DMEM (Sigma-Aldrich) for HBL100, EEMEM (Sigma-Aldrich) for BT-20 and MCF-7 (with 0.01 mg/mL insulin); McCoy (Sigma-Aldrich) for SKBR3 (with 1.5 mmol/L L-glutamine); L-15 (Roche, Basel, Switzerland) for MDA-MB-231 and MDA-MB-435S; MEGM (Cambrex Bio Science) for HMEC. Each medium was supplemented with 10% fetal bovine serum (Cancera International, Etobicoke, Ontario, Canada) and 1% antibiotic/antimycotic solution (Sigma-Aldrich). MDA-MB-231 and MDA-MB-435S cells were maintained at 37°C in a humidified incubator with 5% CO₂. Tissue samples from surgically resected breast cancers and their corresponding clinical information were obtained from the Department of Breast Surgery, Cancer Institute Hospital, Tokyo, after obtaining written informed consent.

**Semi quantitative reverse transcription-PCR analysis.** We extracted total RNA from each of breast cancer clinical samples and from microdissected cells, respectively, and then did T7-based amplification and reverse transcription as described previously (15). We prepared appropriate dilutions of each single-stranded cDNA for subsequent PCR by monitoring the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a quantitative internal control. The PCR primer sequences were 5'-GGGCTGAACAGGAGGGTTTACTTT-3' and 5'-GGTTGAGCACAGGGTACTTG-3' for GAPDH, and 5'-GAAGCTTTAAAGAGTCCTCCTCTG-3' and 5'-GTGTTTTAAGTCAGCATGACGAGCAG-3' for PBK/TOPK.

**Northern blot analysis.** Total RNAs were extracted from all breast cancer cell lines using RNaseasy kit (Qiagen, Valencia, CA) according to the instructions from the manufacturer. After treatment with DNase I (Nippon Gene, Osaka, Japan), mRNA was isolated with mRNA purification kit (GE Healthcare, Buckinghamshire, United Kingdom) following the instructions of the manufacturer. One microgram each of mRNA isolated from normal adult human mammary gland (Biochain, Hayward, CA), lung, heart, liver, kidney, and bone marrow (BD Biosciences, San Jose, CA) was separated on 1% denaturing agarose gels and transferred to nylon membranes (Breast cancer Northern blots). Human multiple-tissue Northern blots (BD Biosciences) were hybridized with [α-32P]dCTP-labeled PCR products of PBK/TOPK prepared by reverse transcription-PCR (RT-PCR see below) or with [α-32P]dCTP-labeled β-actin as a loading control, respectively. Prehybridization, hybridization, and washing were done according to the recommendations from the supplier. The blots were autoradiographed with intensifying screen at −80°C for 14 days. Specific probe for PBK/TOPK (320 bp) was prepared by RT-PCR using the primer set 5'-AGACCTTCATAAGTGTGCTCCTCTT-3' and 5'-AGACCATCCAAAGTGTGCTCCTCTT-3' (underlines indicate recognition sites of restriction enzymes) for wild-type PBK/TOPK. The PCR products were inserted into the EcouI and XhoI sites of pCAGGS-nHA expression vector. We also did two-step mutagenesis PCR to generate a kinase-dead mutant in which Lys45 and Lys46 were substituted to alanines (K64-65A), as described previously (10). The primer sets used for K64-65A were 5'-CAGAGATTCATGGAAGGGATCAGTAATTTC-3' and 5'-GATATCCGCATCCGGTGTTTCGCCTAGA-3' (underlines indicate nucleotides that were replaced from the wild type). All of the constructs were confirmed by DNA sequencing (ABI3700, PE Applied Biosystems, Foster, CA).

**Cloning and mutagenesis.** To construct the PBK/TOPK expression vectors, the entire coding sequence of PBK/TOPK cDNA was amplified by the PCR using the KOD-Plus DNA polymerase (Toyobo, Osaka, Japan). Primer sets were 5'-CCGAGATTCATGGAAGGGATCAGTAATTTC-3' and 5'-GGTACCCTCAAGGATCTCTTGTTAGTTC-3' (underlines indicate recognition sites of restriction enzymes) for wild-type PBK/TOPK. The PCR products were inserted into the EcouI and XhoI sites of pCAGGS-nHA expression vector. We also did two-step mutagenesis PCR to generate a kinase-dead mutant in which Lys51 and Lys52 were substituted to alanines (K64-65A), as described previously (10). The primer sets used for K64-65A were 5'-CAGAGATTCATGGAAGGGATCAGTAATTTC-3' and 5'-GATATCCGCATCCGGTGTTTCGCCTAGA-3' (underlines indicate nucleotides that were replaced from the wild type). All of the constructs were confirmed by DNA sequencing (ABI3700, PE Applied Biosystems, Foster, CA).

**Immunocytochemical staining.** To examine the subcellular localization of endogenous PBK/TOPK protein in breast cancer cell lines, T47D, BT-20, and HBC5, we seeded the cells at 2 × 10⁶ per well (Lab-Tek II chamber slide, Nalgen Nunc International, Naperville, IL). Forty-eight hours after incubation, cells were fixed with PBS (-) containing 4% paraformaldehyde for 15 minutes, and rendered permeable with PBS (-) containing 0.1% Triton X-100 at 4°C for 2.5 minutes. Subsequently, the cells were covered with 3% bovine serum albumin in PBS (-) at 4°C for 12 hours to block nonspecific hybridization followed by incubation with a mouse anti-PBK/TOPK monoclonal antibody (BD Biosciences) diluted at 1:100. After washing with PBS (-), the cells were stained by an Alexa Fluor-448-conjugated anti-mouse secondary antibody (Molecular Probes, Eugene, OR) diluted at 1:1,000. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Fluorescent images were obtained under a TCS SP2 AOBs microscope (Leica, Tokyo, Japan). To examine phosphorylated histone H3 at Ser10 (Ser10)-specific rabbit polyclonal antibody (Cell Signaling Technologies, Beverly, MA).

**Immunohistochemical staining.** Expression patterns of PBK/TOPK protein in breast cancer and normal tissues were investigated as described previously (7) by using anti-PBK/TOPK mouse monoclonal antibody (BD Biosciences). For investigation of normal organs, we purchased commercially available tissue sections of heart, lung, liver, kidney, and testis (Biochain). Briefly, paraffin-embedded specimens were treated with xylene and ethanol, and were blocked by protein-blocking reagent (DakoCytonation, Carpinteria, CA). The monoclonal antibody in antibody-diluted solution (1:50) was added and then stained with substrate-chromogen (DAKO liquid 3,3′-diaminobenzidine chromogen, DakoCytonation). Finally, tissue specimens were stained with hematoxylin to discriminate nuclear from cytoplasm.
interference (RNAi) expression system using psiU6BX3.0 small interfering RNA (siRNA) expression vector (16). An siRNA expression vector against PBK/TOPK (psiU6BX3.0-PBK/TOPK) was prepared by cloning of double-stranded oligonucleotides into the BbsI site in the psiU6BX3.0 vector (Table 1). All of the constructs were confirmed by DNA sequencing.

**Gene-silencing effect by siRNA.** Human breast cancer cell lines, T47D and BT-20, were plated onto 15 cm dishes (4 × 10⁶ per dish) and transfected with 16 μg each of psiU6BX3-Mock (without insertion) and psiU6BX3-PBK/TOPK (si-1, si-2, si-3, and constructs including three-base substitutions in si-3; Table 1) using FuGENE6 reagent (Roche) according to the instructions from the manufacturer. At 24 hours after the transfection, cells are seeded for colony formation assay (2 × 10³/10 cm dish), RT-PCR (2 × 10⁷/10 cm dish), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT; 1 × 10⁵ per well). We selected the psiU6BX3.0-introduced T47D or BT-20 cells with medium containing 0.7 or 0.6 mg/mL neomycin (Geneticin, Invitrogen, Carlsbad, CA), respectively. We changed culture medium twice a week. Total RNAs were extracted from the cells at 11-day incubation with neomycin, and then the knockdown effect of siRNAs was examined by a semiquantitative RT-PCR using specific primer sets; 5′-ATGGAATCCATATACCACCTCT-3′ and 5′-GGTTGACAGGG-TACTTATT-3′ for GAPDH as an internal control, and 5′-GCTTCTCAT-CATCCAACATT-3′ and 5′-GGCAAATATGTCTGCCTTGT-3′ for PBK/TOPK. Transfectants expressing siRNA were grown for 3 weeks in selective medium containing neomycin, then fixed with 4% paraformaldehyde for 15 minutes before staining with Giemsa solution (Merck, Whitehouse Station, NJ) to assess colony number. To quantify cell viability, MTT assays were done with cell counting kit-8 according to recommendations from the manufacturer (Wako, Osaka, Japan). Absorbance at 570 nm wavelength was measured with a Microplate Reader 550 (Bio-Rad). These experiments were done in triplicate.

**In vitro and in vivo kinase assay.** To evaluate the kinase activity of PBK/TOPK, we did in vitro kinase assay using full-length recombinant protein of PBK/TOPK (Invitrogen). Briefly, 1 μg PBK/TOPK protein was incubated in 30 μl kinase assay buffer [50 mmol/L Tris-HCl (pH 7.5)/150 mmol/L NaCl/10 mmol/L MgCl₂/10 mmol/L NaF/1 mmol/L Na₃VO₄/1 mmol/L EDTA/1 mmol/L DTT/50 mmol/L ATP] and then supplemented with 5 μCi of [γ-³²P]ATP (GE Healthcare). For the substrates, we added 5 μg histone mixture or 2.5 μg histone H3 (Roche) in the reaction solutions. After 30-minute incubation at 30°C, the reactions were terminated by addition of SDS sample buffer. After boiling, the protein samples were electrophoresed on 10% to 20% gradient gel (Bio-Rad), and then autoradiographed. To further examine whether histone H3 (Ser³⁸⁰) was phosphorylated by PBK/TOPK, we transfected the wild-type protein and kinase-dead mutant (K64-65A) into T47D cells. After 48-hour culture, we treated 100 nmol/L okadaic acid for 6 hours to activate PBK/TOPK. A total amount of H3 protein as well as the level of its phosphorylation were examined with anti-histone H3 (Abcam, Cambridge, United Kingdom) and anti-phospho-H3 (Ser³⁸⁰) antibody (Cell Signaling Technologies), respectively.

**Results**

**Up-regulation of PBK/TOPK in breast cancers.** We previously did genome-wide expression profile analysis of 81 breast cancer cases using cDNA microarray (8). Among genes up-regulated in breast cancers, we searched genes that encode proteins containing a kinase domain, either on the basis of reported information or according to prediction by protein-motif program SMART¹ and focused on PBK/TOPK for which the high level of transactivation was confirmed in the great majority of breast cancer cells (Fig. 1A).

Northern blot analysis of 10 breast cancer cell lines and six normal organs further confirmed that PBK/TOPK was specifically up-regulated in all of the 10 breast cancer cell lines examined, but its expression was hardly detectable in lung, heart, liver, kidney, bone marrow, and mammary gland (Fig. 1B). To further examine expression pattern of PBK/TOPK in various normal tissues, we did Northern blot analysis using mRNAs from 23 tissues and identified two transcripts exclusively in the testis and thymus (Fig. 1C). According to the National Center for Biotechnology Information database, two representative transcripts of 1,899 nucleotides (Genbank accession no. NM018492) and 1,548 nucleotides (Genbank accession no. AF189722) that share the same open reading frame encoding a 322-amino-acid peptide seemed to correspond to the two bands observed in Northern blot analysis.

**Immunocytochemical and immunohistochemical analysis of PBK/TOPK.** We investigated endogenous expression of PBK/TOPK protein in cell lysates from breast cancer cell lines, BT-20, HBC4, HBC5, HBL100, MCF-7, MDA-MB-231, SKBR3, and T47D by Western blot analysis (Fig. 2A), using HMEC as a control of the experiments. All breast cancer cell lines showed high levels of PBK/TOPK expression, whereas HMEC cells showed no expression. Subsequent immunocytochemical analysis of breast cancer cell lines, T47D, BT-20, and HBC5, using anti-PBK/TOPK monoclonal antibody indicated the localization of endogenous PBK/TOPK mainly in cytoplasm (Fig. 2B). To further investigate PBK/TOPK expression in breast cancer and normal tissue sections, we did immunohistochemical staining with anti-PBK/TOPK monoclonal antibody. We identified strong staining in the cytoplasm of three different histologic subtypes of breast cancer—intraductal carcinoma, papillotubular carcinoma, and scirrhus carcinoma (Fig. 2C–E)—but its expression was hardly detectable in normal breast tissues (Fig. 2F). Furthermore, in concordance with the results of Northern blot analysis, its strong staining was detected at outer cell layer of seminiferous tubules of testis, whereas no expression was observed in any of heart, lung, liver, and kidney (Fig. 3A–F).

**Knockdown effects of endogenous PBK/TOPK.** To investigate its growth-promoting role in breast cancer cells, we knocked down the expression of endogenous PBK/TOPK in two breast cancer cells, T47D and BT-20 (Fig. 4A and B), by means of the RNAi technique. Semiquantitative RT-PCR experiments detected

**Table 1. Target sequences of PBK/TOPK inserted into siRNA expression vector**

<table>
<thead>
<tr>
<th>psiU6BX3.0-si-#1</th>
<th>5′-GGCACATATTGCGTCCCTT-3′</th>
</tr>
</thead>
<tbody>
<tr>
<td>psiU6BX3.0-si-#2</td>
<td>5′-CTGGATGATCATACCTACAGA-3′</td>
</tr>
<tr>
<td>psiU6BX3.0-si-#3</td>
<td>5′-CTGGGCTGGCTAATAAAA-3′</td>
</tr>
<tr>
<td>psiU6BX3.0-mis-#1</td>
<td>5′-GCCTTGCTTTCGAAATA-3′</td>
</tr>
<tr>
<td>psiU6BX3.0-mis-#2</td>
<td>5′-GCCTTGCTTTCGAAATA-3′</td>
</tr>
<tr>
<td>psiU6BX3.0-mis-#3</td>
<td>5′-GCCTTGCTTTCGAAATA-3′</td>
</tr>
</tbody>
</table>

NOTE: Underlined bases indicate siRNA-targeting sequences designed from PBK/TOPK mRNA (Genbank accession no. NM018492). *Mismatched oligonucleotides were designed from si-3 by substitution of some internal bases (indicated by small letters).

¹ http://smart.embl-heidelberg.de.
significant knockdown effect of PBK/TOPK in the cells transfected with PBK/TOPK si-#2 and si-#3, but not with PBK/TOPK si-#1 or control siRNA (mock). In concordance with the knockdown effect, colony formation and MTT assays clearly revealed growth suppression of breast cancer cells by the two siRNAs, PBK/TOPK si-#2 and si-#3, compared with two siRNAs showing no knockdown effect. To exclude a possibility of off-target effect by PBK/TOPK siRNA (si-#3), we generated three additional siRNAs, each of which contained three-base replacement in PBK/TOPK si-#3 (mis-#1, mis-#2, and mis-#3; Table 1). These constructs showed no suppressive effect on the PBK/TOPK expression (Fig. 4C) or on the growth of breast cancer cells. These findings implied a critical role of PBK/TOPK in the growth of breast cancer cells.

In addition, we noticed phenotypic alterations of the cells transfected with siRNAs showing the significant knockdown effect. We observed prolonged midbodies as well as incorrect cell divisions by abnormal cytokinesis in T47D cells in which PBK/TOPK expression was suppressed (Fig. 4D and E). Western blot and fluorescence-activated cell sorting (FACS) analyses also identified an increase in the population of apoptotic (sub-G1) cells in the cells treated with PBK/TOPK siRNA (31.91%), although no phenotypic alteration or increase of sub-G1 population was observed in those transfected with mock construct (5.95%; Fig. 4F and G), implying indispensable roles of PBK/TOPK on proliferation as well as on mitosis and/or cytokinesis for breast cancer cells.

**Cell cycle–dependent expression of PBK/TOPK.** Because PBK/TOPK was reported to be a possible mitotic kinase (10), we investigated its relation to the cell cycle progression. We examined expression of PBK/TOPK protein in T47D cells after synchronization of cell cycle by aphidicolin. FACS analysis showed that the proportion of the cells at G2-M phase was significantly increased at 9 hours (63.94%) after the release from the cell cycle arrest (Fig. 5A). Interestingly, Western blot analysis detected an additional band of high molecular weight of PBK/TOPK at 9 to 12 hours later when most of the cells were at G2-M phase. The intensity of the high-molecular band was decreased at the 15-hour point (Fig. 5B). Immunochemical analysis also revealed the subcellular localization of PBK/TOPK protein around the condensed chromosome in the cells at mitosis, especially at prophase and metaphase (Fig. 5C).

To further investigate a role of high-molecular PBK/TOPK in cell cycle progression, we treated T47D breast cancer cells with nocodazole, and then did Western blot and FACS analyses. As expected, an additional high-molecular band of the endogenous PBK/TOPK in T47D cells was elevated in time-dependent manner.
After treatment of nocodazole (Fig. 5D, left), and was disappeared by treatment of λ phosphatase (Fig. 5D, right). In addition, FACS analysis showed that the proportion of the cells at G2-M phase was elevated from 6 to 18 hours after treatment of nocodazole (Fig. 5E), indicating an important role of phosphorylated PBK/TOPK in mitosis.

**PBK/TOPK phosphorylated histone H3 (Ser^{10}) in vitro and in vivo.** Because PBK/TOPK protein was localized mainly around chromosomal surfaces in mitotic cells, particularly at prophase and metaphase, we focused on histone as a candidate substrate for PBK/TOPK protein. We did in vitro kinase assay using purified recombinant PBK/TOPK and mixed histone proteins (H1a, H2a, H3, and H4; Fig. 6A), and detected ~15 kDa of phosphorylated protein (lane 2), indicating that PBK/TOPK protein might phosphorylate histone H3 protein on the basis of its molecular size (Fig. 6A, left). We further did in vitro kinase assay using histone-H3 recombinant protein and confirmed that PBK/TOPK protein phosphorylated histone H3 (Fig. 6A, right). In addition, we detected an ~40 kDa of autophosphorylated PBK/TOPK by in vitro kinase assay as shown in Fig. 6A (asterisk).

Localization of PBK/TOPK around chromosome as well as its elevated phosphorylation in the early stage of mitosis allowed us to investigate the physiologic role of histone H3 phosphorylation by PBK/TOPK in breast cancer cells. We first transfected wild-type or kinase-dead (K64-65A) PBK/TOPK into T47D cells and then stimulated the cells by treatment of okadaic acid, which is known to induce premature mitosis (10). Both wild-type and kinase-dead PBK/TOPK were phosphorylated at the same level with okadaic acid treatment by Western blot analysis using antihemagglutinin rat antibody. However, the wild-type protein enhanced phosphorylation of histone H3 at Ser^{10} compared with kinase-dead mutant protein (Fig. 6B). Additionally, we confirmed that phosphorylation of histone H3 at Ser^{10} was specifically reduced in PBK/TOPK-depleted T47D cells by siRNA (si-#3), compared with in mock- or mis-1-siRNA-transfected cells (Fig. 6C).

In addition, we examined localization of endogenous PBK/TOPK protein and phosphorylated histone H3. We synchronized T47D and HBC5 cells with aphidicolin, and then did immunocytochemical staining using both anti-PBK/TOPK and anti-phospho-Ser^{10} H3 antibodies. As shown in Fig. 6D, we observed

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**Figure 2.** Expression of PBK/TOPK in breast cancer cell lines and tissue sections. A. expression of endogenous PBK/TOPK protein in breast cancer cell lines in comparison with HMEC cell line, examined by Western blot analysis using anti-PBK/TOPK monoclonal antibody. B. Three breast cancer cell lines, T47D, BT-20, and HBC5, were immunocytochemically stained with anti-PBK/TOPK monoclonal antibody (red) and DAPI (blue) to discriminate nucleus (see Materials and Methods). The endogenous PBK/TOPK protein was stained at the cytoplasm. C to F, immunohistochemical staining results of breast cancer (C-E) and normal breast (F) tissue sections. Endogenous PBK/TOPK protein was stained by use of anti-PBK/TOPK monoclonal antibody. The expression was hardly detected from normal breast tissues (F), but cancer cells were intensely stained at cytoplasm in all cancer tissues investigated, including intraductal (C), papillotubular (D), and scirrhous carcinoma (E). Original magnifications, ×100 (left) and ×200 (right).
partial overlapping of PBK/TOPK and phosphorylated histone H3 around condensed chromosome in prophase cells, and overlapping of both proteins in metaphase cells as well (Fig. 6E; yellow arrow) but their disappearance at anaphase (Fig. 6F; yellow arrow). Taken together, we concluded that endogenous PBK/TOPK is able to specifically phosphorylate histone H3 at Ser10 during mitosis, especially prophase to metaphase in breast cancer cells.

Discussion

cDNA microarray techniques have shown its great potential to effectively generate comprehensive information for screening of novel molecular targets that are useful for development of novel therapeutic reagents (2). In conventional drug screening approaches, the great majority of drug candidates that enter into clinical trials fail in development due to the adverse reactions or the insufficient efficacy. To reduce the failure risk during clinical trials, selection of molecules that are applicable for screening of small molecular compounds is critically important. In this point of view, we have constructed the expression profile database of cancer cells as well as >29 normal human tissues and have been taking a strategy to select genes that are frequently overexpressed in cancer cells, but not expressed in normal organs. Through this selection process, we have chosen dozens of genes, and subsequently confirmed overexpression of candidate genes by semiquantitative RT-PCR and Northern blot analyses. Among the validated genes, we focused on PBK/TOPK, which was overexpressed in breast cancers and not expressed in 29 normal human tissues examined except testis and thymus. The immunohistochemical analysis also supported the high level of endogenous PBK/TOPK expression in concordance with the results of Northern blot analysis. In addition, we showed by means of the siRNA technique that knocking down the expression of endogenous PBK/TOPK resulted in growth suppression of breast cancer cell lines (Fig. 4A-C). Furthermore, we have shown that PBK/TOPK was also commonly up-regulated in bladder cancers and non–small cell lung cancers as well as breast cancers through cDNA microarray data (data not shown). Taken together, these findings suggest that PBK/TOPK has an oncogenic role, not only in breast cancers but also in bladder cancers and non–small cell lung cancers.

PBK/TOPK was indicated to be involved in mitosis as shown by its significant role in highly proliferating spermatocytes (10, 17). Our immunohistochemical analysis of testis confirmed the expression of PBK/TOPK around the outer region of seminiferous tubules where repeated mitosis of sperm germ cells followed by meiosis occurs (17). In addition to important roles of PBK/TOPK in testis, our findings of its subcellular translocation during M phase indicated its critical function at mitosis in cancer cells. Moreover, we showed that knockdown of PBK/TOPK expression with specific siRNAs caused dysfunction of cytokinesis and subsequently led to apoptosis of the cancer cells (Fig. 4D-G). These results suggest that PBK/TOPK is likely to play an important role in cell division and cytokinesis. It is notable that microscopic and FACS observations for the siRNA effect of PBK/TOPK are quite similar with those of Annexin 11, which is required for cytokinesis completion; knockdown of Annexin 11 resulted in narrow cytoplasmic bridge and increased population of cells at sub-G1 (18).
Because PBK/TOPK was indicated to contain a kinase domain, we treated the cells with several kinds of stimuli, including okadaic acid, phorbol 12-myristate 13-acetate, \( \beta \)-estradiol, and nocodazole, to investigate its relationship with estrogen receptor and cell mitotic signals, respectively (data not shown). Among these stimuli, we found phosphorylation of PBK/TOPK after the treatment with okadaic acid that is a specific inhibitor of serine/threonine protein phosphatase and also known to cause mitosis-like processes in interphase cells, chromosome condensation, and entry into mitosis in the Cdc2-independent manner (19, 20). In a previous study, PBK/TOPK was indicated to be a MAPKK-like protein that phosphorylated the p38 protein (11). In addition, the possible interaction between Raf and PBK/TOPK was shown by the yeast two-hybrid screening analysis (21). These two findings implied that PBK/TOPK might be involved in the MAPK pathway. However, our results of in vitro kinase assay failed to show that PBK/TOPK is an upstream kinase of the p38 and p42/extracellular signal-regulated kinase 2 proteins, which were up-regulated commonly in breast cancer cell lines (data not shown). Instead, we first observed phosphorylation of histone H3 by PBK/TOPK with high selectivity. Interestingly, phosphorylation at the NH\(_2\) terminus of histone H3 (Ser\(^{16}\)) is indicated to be an early mitotic event, accompanied with chromosome condensation after okadaic acid treatment (19). Because our immunostaining experiment using breast cancer cells revealed subcellular localization of PBK/TOPK around the chromosome in the cells at mitosis, especially at prophase and metaphase, we hypothesized that PBK/TOPK might be involved in the mitotic process at metaphase and that PBK/TOPK might be involved in the mitotic process at metaphase and anaphase.

![Figure 4. Growth-inhibitory effects of PBK/TOPK siRNAs in breast cancer cell lines, T47D and BT-20.](image)

*Figure 4.* Growth-inhibitory effects of PBK/TOPK siRNAs in breast cancer cell lines, T47D and BT-20. Semiquantitative RT-PCR results showed PBK/TOPK silencing at 11 days after neomycin selection. GAPDH served as an internal control. MTT assays were done to evaluate cell viability at 11 days and graphed after standardization by mock to 1.0. Colony formation assays were carried out 3 weeks after selection (see Materials and Methods). Two siRNA constructs (si-#2 and si-#3) showed knockdown effects against internal PBK/TOPK expression and inhibited cell growth in both T47D (A) and BT-20 (B). PBK/TOPK-specific targeting was further confirmed by generation of mismatched siRNAs (mis-#1, mis-#2, and mis-#3; originally designed from si-#3) in T47D (C). Mock was used for a negative control. The phenotypic differences between mock control (D) and si-#3–induced T47D cells (E) were investigated by microscopic observation, 2 weeks after neomycin selection. Irregular appearances of cell were observed from PBK/TOPK-depleted cells, prolonged midbody, abolished, and uncontrolled cytokinesis (E). F, silencing of internal PBK/TOPK expression by siRNA (si-#3) was confirmed by Western blotting. G, FACS results showed more population of apoptotic cells (represented by sub-G\(_1\) percentage) in si-#3–induced T47D cells (si-#3) rather than mock control. A total of 10,000 cells were equally counted from mock- and si-#3–transfected T47D cells.
metaphase (Fig. 5C), we examined whether PBK/TOPK may phosphorylate histone H3 at Ser\textsuperscript{10} in vivo. A comparison of the wild-type and kinase-dead PBK/TOPK protein with/without okadaic acid stimulation showed that PBK/TOPK phosphorylated at Ser\textsuperscript{10} of histone H3 (Fig. 6B), and endogenous PBK/TOPK protein was well merged with phosphorylated histone H3 in mitotic cells (Fig. 6D). Posttranslational modifications at the NH\textsubscript{2}-terminal portion of histone H3, including acetylation, methylation, and phosphorylation, were described previously (22–24). Among them, phosphorylation of histone H3 at Ser\textsuperscript{10} is involved in the initiation of mammalian chromosome condensation, an essential event in cell mitosis (24, 25). According to the “ready production label” model, Ser\textsuperscript{10} phosphorylation of histone H3 reaches at the maximum level in metaphase, as an indication that the chromosomes are ready to be separated, and then Ser\textsuperscript{10} is dephosphorylated accompanied by metaphase/anaphase transition (26). Cell cycle–dependent Ser\textsuperscript{10} phosphorylation of histone H3 is well correlated with PBK/TOPK expression level and localization, particularly in the early stage of mitosis (Fig. 6D and E). Therefore, we suggest that PBK/TOPK–histone H3 pathway may promote mitotic events, thus enhancing cancer cell proliferation, similarly to Pak1 whose significant role is indicated in breast cancer cells (27). However, morphologic changes of the cells in which PBK/TOPK was knocked down by siRNA implied a presence of other substrates that might be involved in cytokinesis (Fig. 4). In this study, we used okadaic acid, which is known to induce premature mitosis, as a stimulus for activation of PBK/TOPK. Interestingly, previous reports indicated that okadaic acid induced Ser\textsuperscript{10} phosphorylation of histone H3 through inhibition of protein phosphatases (28, 29). For example, Aurora-A is deactivated by protein phosphatase 2A (PP2A), but to be reactivated by its autophosphorylation through binding with TPX2 (targeting protein for Xenopus kinesin-like protein 2) protein that impairs the activity of PP2A (29).

PBK/TOPK has been reported as a member of the Ser/Thr kinase family. Our results presented here indicated that PBK/TOPK is overexpressed in breast cancer and its kinase activity possibly plays a significant role in breast cancer cell growth. Recent drug development is focused on targeting important
molecules involved in the oncogenic pathways, represented by imatinib mesylate and trastuzumab. The combination of breast cancer expression profiling with RNAi should help us to identify such drug targets for therapy (30). The present results of kinase activity further provide promising possibilities to develop novel anticancer agent.

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