Plakophilin 3 Oncogene as Prognostic Marker and Therapeutic Target for Lung Cancer

Chiyuki Furukawa,1,2 Yataro Daigo,1 Nobuhisa Ishikawa,1 Tatsuya Kato,1 Tomoo Ito,3 Eiju Tsuchiya,1 Saburo Sone,1 and Yusuke Nakamura1

1Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo; 2Department of Internal Medicine and Molecular Therapeutics, The University of Tokushima School of Medicine, Tokushima; and 3Department of Surgical Pathology, Hokkaido University Hospital, Sapporo; and Kanagawa Cancer Center Research Institute, Kanagawa, Japan

Abstract

We investigated gene expression profiles of non–small cell lung carcinomas (NSCLC) to screen candidate molecules that might be useful as diagnostic markers or for development of novel molecular-targeting therapies. Here we report evidence that a member of the armadillo protein family, plakophilin 3 (PKP3), is a potential molecular target for treatment of lung cancers and might also serve as a prognostic indicator. We documented elevated expression of PKP3 in the great majority of NSCLC samples examined. Treatment of NSCLC cells with small interfering RNAs of PKP3 suppressed growth of the cancer cells; on the other hand, induction of exogenous expression of PKP3 conferred growth-promoting activity on COS-7 cells and enhanced their mobility in vitro. To investigate its function, we searched for PKP3-interacting proteins and identified dynamin 1-like, which was also activated in NSCLC. In addition, a high level of PKP3 expression was associated with poor survival as well as disease stage and node status for patients with lung adenocarcinoma, suggesting an important role of the protein in development and progression of this disease. As our data imply that up-regulation of PKP3 is a frequent and important feature of lung carcinogenesis, we suggest that targeting the PKP3 molecule might hold promise for development of a new therapeutic and diagnostic strategy for clinical management of lung cancers. (Cancer Res 2005; 65(16): 7102-10)

Introduction

Lung cancer is the leading cause of cancer deaths worldwide, and non–small cell lung cancer (NSCLC) accounts for nearly 80% of those cases (1). Many genetic alterations associated with development and progression of lung cancer have been reported, but the precise molecular mechanisms remain unclear (2). Within the last decade several newly developed cytotoxic agents such as paclitaxel, docetaxel, gemcitabine, and vinorelbine have begun to offer multiple choices for treatment of patients with advanced lung cancer; however, each of those regimens confers only a modest survival benefit compared with cisplatin-based therapies (3, 4). Hence, novel therapeutic strategies such as molecular-targeted drugs and antibodies and cancer vaccines are eagerly being sought.

Systematic analysis of expression levels of thousands of genes in tumors is an effective approach for identifying unknown molecules involved in processes of carcinogenesis and for selecting candidates for development of novel anticancer drugs and tumor markers (5–12). We have been attempting to isolate potential molecular targets for diagnosis and treatment of lung cancer by analyzing genome-wide expression profiles of NSCLC cells on a cDNA microarray containing 23,040 genes, using pure populations of tumor cells prepared from 37 cancer tissues by laser capture microdissection (5). To verify the biological and clinicopathologic significance of the respective gene products, we have been doing tumor tissue microarray analysis of clinical lung cancer materials (11, 12). In the course of those systematic studies, we observed that the gene encoding plakophilin 3 (PKP3) was frequently trans-activated in primary NSCLCs.

PKP3 is a member of the p120ctn/plakophilin subfamily of armadillo (ARM) proteins that are synthesized in cells of stratified and single-layered epithelia; armadillo molecules provide physical links between selectively synthesized desmosomal proteins (13–15). Whereas ARM-related proteins in general have structural roles in cell contact and cytoskeleton-associated activities, they can also exert signaling functions by generating and transducing signals that affect gene expression. Multiple studies have implied that genetic aberrations in members of the ARM-protein family, including plakoglobin (PKGB), β-catenin (CTNNB1), and adenomatous polyposis coli (APC), contribute to tumor development and/or progression (16, 17). However, the role(s) of subfamily member PKP3 during carcinogenesis, or even its function in normal epithelial cells, have not been clarified.

Here we report evidence that recommends PKP3 as a potential target for development of novel therapeutic drugs and diagnostic/prognostic markers for lung cancer and that suggests possible molecular roles of this protein through interaction with dynamin 1-like (DNM1L) in carcinogenesis and progression of NSCLC.

Materials and Methods

Cell lines and clinical tissue samples. The human NSCLC cell lines used in this study were as follows: adenocarcinoma represented by A427, A549, LC319, PC3, PC9, PC14, NCI-H1373, NCI-H1666; squamous cell carcinoma (SCC) BERR-LC-AL, SK-MES-1, EBC-1, LU61, NCI-H226, NCI-H520, NCI-H647, NCI-H1703, NCI-H2170; and large cell carcinoma (LCC) LX-1. A human bronchial epithelial cell line, BEAS2B was included in the panel of the cells used in this study. All cells were grown in monolayers in appropriate medium supplemented with 10% FCS and were maintained at 37°C in an atmosphere of humidified air with 5% CO2.

Primary NSCLC samples, of which 22 were classified as adenocarcinomas, 14 as SCCs, and one as adenosquamous carcinoma (ASC), had been obtained from 37 patients, with written informed consent, for a
study described previously (5). Sixteen additional primary NSCLCs, including eight adenocarcinomas and eight SCCs, were obtained along with adjacent normal lung tissue samples from new patients undergoing surgery at the Hokkaido University and its affiliated hospitals (Sapporo, Japan).

A total of 293 formalin-fixed samples of primary NSCLCs (stage I-IIIA) including 160 adenocarcinomas, 102 SCCs, 17 LCCs, 10 bronchioalveolar cell carcinomas (BACs), 4 ASCs, and adjacent normal lung tissue, had been obtained earlier along with clinicopathologic data from patients who had undergone surgery at the Hokkaido University and its affiliated hospitals. Tumor-specific clinical follow-up data were available for 279 of those patients.

**Semiquantitative reverse transcription-PCR analysis.** Total RNA was extracted from cultured cells and clinical tissues using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol. Extracted RNA and normal human tissue polyadenylate RNA were treated with DNase I (Nippon Gene, Tokyo, Japan). Extracted RNAs were reversely transcribed using oligo(dT)$_{18}$ primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Semiquantitative reverse transcription-PCR (RT-PCR) was carried out with synthesized gene-specific primers PKP3 (5′-AGCCTGGAGAAGGGCTAAT-3′ and 5′-AGTGGCTGCTG-ATGCAAGATGACT-3′), PKP1 (5′-CTCAGACAGCCGTTTGTAGAC-3′ and 5′-GTGAACTCTCCCAAATCTGAC-3′), PKP2 (5′-GCAAGAGGAAT- TAAGGCTCTTCA-3′ and 5′-CTGCTGGATCTGTCGACATCTTCT-3′), desmoglein 1 (DSG1; 5′-GAGGTGTTTAGGATATACACTCG-3′ and 5′-CAAATCTCCGCTCTCTATG-3′), desmoplakin (DSP; 5′-GCTCTC- ATGATAGGATATAGATC-3′ and 5′-AGAAGTAGAATGTCCG-3′), NMIL (5′-GGAGACCTCTTCTTGGAGGAG-3′ and 5′-AAGGACGACGT- CAATATTGAT-3′), and ACTB (5′-GGAGGTAGTACGTTCTTGCTG-3′ and 5′-CAAGCTGACCTGTCAGAAG-3′) as internal controls. PCR reactions were optimized for the number of cycles to ensure product intensity within the logarithmic phase of amplification. All PCR reactions involved initial denaturation at 94°C for 2 minutes followed by 22 (for ACTB) or 30 to 35 cycles (for other genes) of 94°C 30 seconds, 54°C to 60°C for 30 seconds, and 72°C for 60 seconds on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA).

**Northern blot analysis.** Human multiple-tissue blots (BD Biosciences, Clontech, Palo Alto, CA) covering 16 tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, colon, and peripheral blood leukocyte) were hybridized with a 32P-labeled PCR product of PKP3. The full-length cDNA of PKP3 was prepared by RT-PCR using primers 5′-CGCCGATCTCCATG-GAGGAGCTTAATGCTG-3′ and 5′-GTCCTGCTCTCTCTCGATG-3′. Prehybridization, hybridization, and washing were done according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at -80°C for 96 hours.

**Western blotting.** Cells were lysed in Iysis buffer: 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, plus protease inhibitor (Protease Inhibitor Cocktail Set III; Calbiochem, Darmstadt, Germany). We used an enhanced chemiluminescence Western blotting analysis system (Amersham Biosciences, Piscataway, NJ), as previously described (12). A mouse monoclonal antibody to human PKP3 (Zymed Laboratories, Inc., South San Francisco, CA) was proved to be specific to human PKP3 and have no cross-reactivity with other homologous proteins of human PKP3 and PKP2 (14, 15). To validate our RNA knockdown results, we used Western blot analysis using lysates of NSCLC cells that expressed either of endogenous PKP3, PKP1, and or/and PKP3 (see Fig. 1B).

**Immunocytochemistry.** Cultured cells were washed twice with PBS (−), fixed in 4% formaldehyde solution for 60 minutes at room temperature, and rendered permeable by treatment for 1.5 minutes with PBS (−) containing 0.1% Triton X-100. Cells were covered with 3% bovine serum albumin in PBS (−) for 60 minutes to block nonspecific binding before the primary antibody reaction. Then the cells were incubated with antibody to human PKP3 protein that had been tagged with c-myc. The immune complexes were stained with a goat anti-mouse secondary antibody conjugated to FITC (Cappel, Durham, NC) and viewed with a laser confocal microscope (TCS SP2 AOBs; Leica Microsystems, Wetzlar, Germany).

**Immunohistochemistry and tissue microarray.** To investigate the presence of PKP3 protein in clinical samples (normal lung tissues and NSCLCs that had been embedded in paraffin blocks), we stained the sections using ENVISION+ Kit/ horseradish peroxidase (HRP; DakoCyto- mation, Glostrup, Denmark). Briefly, a mouse monoclonal antibody specific to human PKP3 (Zymed Laboratories) was added after blocking endogenous peroxidase and proteins, and the sections were incubated with HRP-labeled anti-mouse IgG as the secondary antibody. Substrate chromogen was added and the specimens were counterstained with hematoxylin.

Tumor tissue microarrays were constructed using the 293 formalin-fixed NSCLCs described previously (18–21). Each area for sampling was selected on the basis of visual alignment with the corresponding H&E-stained section on a slide. Three, four, or five tissue cores (diameter, 0.6 mm; height, 3-4 mm) taken from each donor tumor block were placed into a recipient paraffin block by means of a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). A core of normal tissue was punched from each case, and 5-μm sections of the resulting microarray block were used for immunohistochemical analysis.

PKP3 positivity was assessed semiquantitatively according to staining intensity as absent (scored as 0), weak (scored as 1+), or strongly positive (scored as 2+) by three independent investigators without prior knowledge of the clinical follow-up data. Cases were accepted only as strongly positive if reviewers independently defined them as such. We used contingency tables to analyze the relationship of PKP3 expression to gender, age, tumor size (pT), and lymph node metastasis (pN) in NSCLC patients; correlation with patient survival was assessed by the Kaplan-Meier method. Statistical differences between the groups were determined with the log-rank test. A total of 279 patients (155 adenocarcinomas, 95 SCCs, 15 LCCs, 10 BACs, and 4 ASCs) were evaluated from the time of surgery to the last known follow-up.

**RNA interference assay.** Using the vector-based RNA interference (RNAi) system, psiH1B3x3, which we had established earlier to directly the synthesis of small interfering RNAs (siRNA) in mammalian cells (8, 12), we transfected 10 μg of siRNA expression vector with 30 μL of LipofectAMINE 2000 (Invitrogen) into three NSCLC cell lines (A549, LC319, and RERF-LC-AI) that endogenously overexpressed PKP3. The transfected cells were cultured for 5 days in the presence of appropriate concentrations of gene silencer (Genie). Cell numbers and viability were measured by Giemsa staining and (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in triplicate. The target sequences of the synthetic oligonucleotides for RNA were as follows: control 1 [enhanced green fluorescent protein (EGFP) gene], a mutant of *Arquerea victoria GFP*), 5′-GAAGGACGACGACTCTTTG-3′; control 2 (Scramble; chloroplast *Euglena gracilis* gene coding for the SS and 16S rRNA), 5′-GCGGCGTTGTTAG-GATTGG-3′; siRNA-PKP3-1 (si-1), 5′-CAGCTACGTTGAGCACCAG-3′; siRNA-PKP3-2 (si-2), 5′-CCTGGTGCCAGTACAACAG-3′. To validate our RNAi system, individual control siRNAs (EGFP and Scramble) were initially confirmed using semiquantitative RT-PCR to decrease expression of the corresponding target genes that had been transiently transfected to COS-7 cells. Down-regulation of PKP3 protein expression by effective siRNA (si-2), but not by controls, was also confirmed with Western blot analysis using anti-PKP3 monoclonal antibody (Zymed Laboratories) in the cell lines above. In this assay, exogenous or endogenous expression of the target gene (EGFP and PKP3) was effectively suppressed in >90% of the transfected cells as examined by immunocytochemical analyses.

**Expression of PKP3 in transfected mammalian (COS-7) cells.** PKP3-expressing stable transfecants were established according to a standard protocol. First, the entire coding region of PKP3 was amplified by RT-PCR using the primer sets described above. The product, digested with EcoRI and XhoI, was cloned into appropriate sites of pcDNA3.1-myc/His A+ vector (Invitrogen) that contained c-myc-His-epitope sequences (LDEE-SILKQE-HHHHHH) at the COOH-terminal of the PKP3 protein. The nucleotide sequences of the DNA clones were determined with an ABI Prism 3700 DNA sequencer (Applied Biosystems), using T7 or synthetic oligonucleotide primers according to the manufacturer's instructions.
To obtain stable transfectants, we transfected COS-7 cells, which scarcely express endogenous PKP3 or most other desmosomal proteins with plasmids expressing PKP3 (pcDNA3.1-PKP3-myc/His) or with mock plasmid (pcDNA3.1), using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. Transfected cells were cultured in DMEM containing 10% FCS and geneticin (0.4 mg/mL) for 14 days; then 50 individual colonies were trypsinized and screened for stable transfectants by a limiting dilution assay. The level of PKP3-myc/His expression was detected in each clone by Western blot analysis and immunostaining.

Cell growth assay. COS-7 transfectants that stably expressed PKP3 were seeded onto 6-well plates (5 × 10⁴ cells per well) and maintained in medium containing 10% FCS and 0.4 mg/mL geneticin for 24, 48, 72, 96, and 144 hours. At each time point, cell proliferation was evaluated by the MTT assay.

Matrigel invasion assay. COS-7 cells transfected with plasmids expressing PKP3, or mock plasmid, were grown to near confluence in DMEM containing 10% fetal bovine serum. The cells were harvested by trypsinization, washed in DMEM without addition of serum or protease inhibitor, and suspended in DMEM at a concentration of 1 × 10⁵ cells/mL. Before the cell suspension was prepared, the dried layer of Matrigel matrix (Becton Dickinson Labware, Bedford, MA) was rehydrated with DMEM for 2 hours at room temperature; then DMEM (0.75 mL) containing 10% FCS was added to each lower chamber of 24-well Matrigel invasion chambers and 0.5 mL (5 × 10⁴ cells) of cell suspension was added to each insert of the upper chamber. The plates of inserts were incubated for 22 hours at 37°C. After incubation, the chambers were processed; cells invading through the Matrigel-coated inserts were fixed and stained by Giemsa as directed by the supplier (Becton Dickinson Labware).

Immunoprecipitation and matrix-assisted laser desorption/ionization-time of flight mass spectrometry mapping of PKP3-associated proteins in non–small cell lung carcinoma cells. Cells were lysed in appropriate amounts of immunoprecipitation buffer (0.5% NP40, 50 mmol/L Tris-HCl, 150 mmol/L NaCl) in the presence of protease inhibitor. To confirm that PKP3 protein is soluble in this extraction condition, both the supernatant and the residual pellet of the lysed cells were obtained after centrifugation, resuspended in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and subsequently served for Western blotting using anti-PKP3 antibody, as described elsewhere (13). By quantifying the signal intensities of the Western blot analysis, we estimated that 24% of endogenous PKP3 in A549 or LC319 cells and 38% of exogenous PKP3 in PKP3-transfected COS-7 cells (PKP3 contained in the supernatant/PKP3 contained in both the supernatant and pellet).

Figure 1. Validation of PKP3 expression in lung cancers. A, expression of PKP3 in clinical samples of NSCLC (T; adenocarcinoma, ADC and SCC) and corresponding normal lung tissues (N), examined by semiquantitative RT-PCR. We prepared appropriate dilutions of each single-stranded cDNA prepared from mRNAs of clinical lung cancer samples, taking the level of β-actin (ACTB) expression as a quantitative control. B, expression of PKP3 protein in NSCLC cell lines, examined by Western blot analysis. C-D, immunohistochemical evaluation of PKP3 expression and localization on lung adenocarcinoma tissues (left, ×100; right, ×400). Positive staining appeared predominantly in the plasma membrane (C) and/or cytoplasm (D).
were detectable in the supernatant fraction. Then, cell extracts from lung cancer cell line LC319 were precleared by incubation at 4°C for 1 hour with 100 μl of protein G-agarose beads, in final volumes of 2 mL of immunoprecipitation buffer in the presence of protease inhibitor. After centrifugation at 1,000 rpm for 5 minutes at 4°C, the supernatants were incubated at 4°C with anti-PKP3 monoclonal antibody (PROGEN Biotechnik, Heidelberg, Germany), which had been confirmed specific to PKP3 (data not shown) or with normal mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 hours. Then, protein G-agarose beads were added into the mixtures and they were additionally incubated at 4°C for 1 hour. The beads were then collected by centrifugation at 5,000 rpm for 2 minutes at 4°C and washed 10 times with 1 mL of each immunoprecipitation buffer. The washed beads were resuspended in 50 μl of Laemmli sample buffer and boiled for 5 minutes before the proteins were separated on 7.5% to 15% SDS-PAGE gels (Bio-Rad Laboratories). After electrophoresis, the gels were stained with silver. Protein bands found specifically in extracts immunoprecipitated with monoclonal antibody to PKP3 were excised to serve for analysis by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS; AXIMA-CFR plus, SHIMADZU BIOTECH, Kyoto, Japan).

We confirmed interaction between endogenous PKP3 and DNM1L (alias dynamin-related protein 1, DRP1), a candidate protein interacting with PKP3 in NSCLC cells, by reciprocal coimmunoprecipitation experiments with extracts from LC319, using anti-PKP3 monoclonal antibody (PROGEN Biotechnik) and anti-DNM1L/DRP1 polyclonal antibody (TREVIGEN, Gaithersburg, MD). To further confirm the interaction between endogenous DNM1L and exogenous PKP3 transfected to COS-7 cells, which were used for several functional assays, we immunoprecipitated the extracts from COS-7 cells transfected with pcDNA3.1-PKP3-myc/His using anti-c-myc monoclonal antibody (Santa Cruz Biotechnology). Immunoblot was done using anti-DNM1L/DRP1 polyclonal antibody.

Results

PKP3 in lung tumors and normal tissues. To search for novel target molecules for development of therapeutic agents and/or diagnostic markers for NSCLC, we first screened genes that showed ≥5-fold higher level of expression in cancer cells than in normal cells, in half or more of the 37 NSCLCs analyzed by cDNA microarray (5). We then compared the expression profile data in NSCLC with those in 31 normal human tissues previously obtained by our group (27 adult and 4 fetal organs; refs. 22, 23) and selected genes according to the following criteria; genes that were specifically expressed in NSCLCs, but their expression was low or hardly detectable in normal tissues. We found that the PKP3 transcript indicating ≥5-fold expression in cancer cells than in normal lung cells (control) in 84% of the NSCLC samples examined and confirmed its elevated expression in 15 of 16 additional NSCLC cases (eight of eight adenocarcinomas and seven of eight SCCs; Fig. 1A). We examined expression of endogenous PKP3 protein in NSCLC cells by Western blot analysis using a mouse monoclonal antibody specific to human PKP3 (Fig. 1B). We also examined expression of PKP3 protein with the same antibody on NSCLC tissues. Positive staining appeared predominantly in the plasma membrane and/or cytoplasm and very weakly (as speckles) in some nuclei (Fig. 1C-D). Northern blotting using PKP3 cDNA as a probe identified the 3.0-kb transcript as a very faint signal and only in placenta, pancreas, and prostate among the 16 normal human tissues examined as concordant to the results of cDNA microarray (data not shown).

Growth inhibitory effect of siRNA designed to reduce expression of PKP3 in non–small cell lung carcinoma cells. To assess whether up-regulation of PKP3 plays a role in growth or survival of lung cancer cells, we designed and constructed plasmids to express siRNA against PKP3 (si-1, si-2), along with two different control plasmids (siRNAs for EGFP or Scramble), and transfected them into A549, LC319, and RERF-LC-AI cells to suppress expression of endogenous PKP3 (representative data was shown in Fig. 2). The amount of PKP3 protein in the cells transfected with si-2 was significantly less than in cells transfected with any of the two control siRNAs (Fig. 2, top): si-1 showed almost no suppressive effect on PKP3 expression. In accord with its suppressive effect on protein expression, transfected si-2 caused significant decreases in colony numbers and cell viability measured by colony formation and MTT assays, but no such effects were observed by two controls or si-1 (Fig. 2, middle and bottom).

Oncogenic effect of PKP3 in mammalian cells. To determine the effect of PKP3 on growth and transformation of mammalian cells, we carried out in vitro assays using COS-7-derived clones that stably expressed PKP3 (COS-7-PKP3). Immunocytochemical analysis using anti-c-myc antibodies detected c-myc- His-tagged PKP3 protein in almost all of the COS-7 cells mainly in the cytoplasm and/or plasma membrane (Fig. 3A). COS-7-PKP3 cells showed a multilayer growth pattern and some of them formed protrusions from the cell body (Fig. 3B). Interestingly, the overexpressed ectopic PKP3 protein was accumulated even in the edge of many protrusions (Fig. 3A), suggesting the possible role of PKP3 in this structural change of COS-7 cells. Using clones stably expressing PKP3 (COS-7-PKP3-1 or COS-7-PKP3-2; Fig. 3C, top), we compared their growth with that of empty vector control clones (COS-7-mock-1 or COS-7-mock-2). Growth of the COS-7-PKP3 cells was promoted in comparison with the empty vector controls (Fig. 3C, bottom), as determined by the MTT assay. There was also a remarkable tendency of COS-7-PKP3 cells to form larger colonies than empty vector clones did.

As COS-7 cells that constitutively expressed PKP3 showed a multilayer growth pattern and formed many protrusions, we did Matrigel invasion assays to determine whether PKP3 might play a role in cellular invasive ability. COS-7-PKP3 cells invaded through the Matrigel significantly more readily than control cells (Fig. 3D).

Identification of DNM1L as a protein interacting with PKP3 in non–small cell lung carcinoma cells. PKP3 is known to interact or colocalize with several desmosomal proteins in epithelial cells (14). To investigate the expression pattern of these desmosomal proteins in NSCLC cells, we analyzed mRNA expression of representative desmosomal components known to be associated with PKP3 (PKP1, PKP2, DSP, and DSG1) by semiquantitative RT-PCR experiments. However, no significant correlation of gene expression between PKP3 and these desmosomal components in the lung cancer samples was observed (Fig. 4A), suggesting that activation of PKP3 could be independent of the PKP3-associated desmosome complex and be mediating growth and/or invasion of lung cancer cells through interaction with other protein(s).

To further elucidate the function of PKP3 in lung cancer, we screened protein(s) that interacts with PKP3 in cancer cells. Lysate of LC319 cells were extracted and immunoprecipitated with monoclonal antibody to PKP3. The protein complex including endogenous PKP3 was stained with SilverQuest (Invitrogen) on the SDS-PAGE gel. An about 87-kDa band, which was seen in immunoprecipitates of cell lysates with anti-PKP3 antibody but not seen in those with normal mouse IgG was extracted and its peptide sequence was determined by MALDI-TOF mass spectrometry. As the peptide sequence matched to human DNM1L, we confirmed interaction of PKP3 with DNM1L by coimmunoprecipitation experiments. To confirm the interaction between endogenous DNM1L and PKP3, we transfected empty vector or PKP3 cDNA as a probe into COS-7 cells. Immunoprecipitation of cell lysates with anti-PKP3 antibody but not with normal mouse IgG was extracted and the protein complex inclu-
spectrometric sequencing. Thus, we identified a protein DNM1L, a member of the dynamin superfamily of large GTPases as a candidate that interacts with PKP3 in lung cancer cells. We confirmed the cognate interaction between endogenous PKP3 and DNM1L in lung cancer cells by immunoprecipitation experiment (Fig. 4B). Moreover, a great majority of the lung cancer cell lines or clinical NSCLC tissue samples as well as COS-7 cells were confirmed to highly express endogenous DNM1L (Fig. 4A, bottom). COS-7 cells were found not to express PKP3 and most of other desmosomal proteins (like PKP1, DSG1, and DSP) except PKP2, consistent with a previous report (14). We further confirmed the cognate interaction between exogenous PKP3 transfected to COS-7 cells used for growth and invasion assays, and endogenous DNM1L protein by immunoprecipitation experiment (data not shown). The results suggested that a PKP3-DNM1L complex might play an important role in lung carcinogenesis.

**Association of high PKP3 expression with disease progression.** We finally examined the correlation between expression of PKP3 protein and clinicopathologic data using tissue arrays consisting of 293 NSCLC tissues. Positive staining appeared in 98% of adenocarcinomas (157 of 160), 97% of SCCs (99 of 102), 94% of LCCs (16 of 17), 100% of BACs (10 of 10), and 100% of ASCs (4 of 4). All of those tumors were surgically resectable NSCLCs, and no staining was observed in any of their adjacent normal lung tissues. We classified a pattern of PKP3 expression on the tissue array ranging from absent/weak (scored as 0 to 1+) to strong (scored as 2+; Fig. 5A). Strong PKP3 staining was not associated with any of the clinicopathologic factors in SCCs. However, in adenocarcinomas, expression levels of PKP3 were significantly associated with node status (N0 versus N1; N2, \( P = 0.0017; \chi^2 \) test; Fig. 5B). The sample sizes of LCCs, BACs, and ASCs were too small to be evaluated further.

We then asked if high level of PKP3 expression would be associated with tumor-specific survival time. Strong PKP3 staining did not correlate with poor tumor-specific survival among SCC patients (\( P = 0.66 \) by the log-rank test), but adenocarcinoma patients whose tumors overexpressed PKP3 suffered shorter tumor-specific survival compared with those with absent/weak PKP3 expression (\( P = 0.009 \) by the log-rank test; Fig. 5C). Using univariate analysis, we found that node status (\( P < 0.0001 \), score test), tumor size (T1 versus T2, T3, T4; \( P = 0.0076 \), score test), and high PKP3 expression (\( P = 0.009 \), score test) were important correllative features for poor prognoses of patients with adenocarcinoma.
Discussion

In recent years, identification and characterization of specific tumor-associated genes and their products have accelerated development of a new type of anticancer agent in the form of molecular-targeted drugs (3, 4, 7, 10), that are highly specific to malignant cells and consequently expected to have minimal adverse effects due to their well-defined mechanisms of action. As an approach to that goal, we have undertaken a strategy that combines screening of candidate molecules by genome-wide expression analysis with high-throughput screening of loss-of-function effects, using antisense S-oligonucleotides and/or the RNAi technique (5–12). In addition, we have been using the tissue microarray method (11, 12) to analyze hundreds of archived clinical samples for validation of potential target proteins. Using this combination of approaches for the work reported here, we determined that PKP3 was overexpressed in the great majority of clinical lung cancer samples and cell lines, and that this gene product is very much involved in promoting growth and progression of lung tumors.

The central domain of PKP3 protein contains 10 Arm repeats, a configuration typical of molecules in the p120ctn/plakophilin subfamily of Armadillo proteins. Arm-repeat proteins are divided into two major categories, the “classic” α-catenin/β-catenin/plakoglobin group and “p120-related” proteins; the latter can be further subdivided into “p120CTN” and “plakophilin” subfamilies. The classic catenins have been studied extensively and their important roles in fetal development and carcinogenesis have been shown. On the other hand, the first clues to the biological function of PKP3 emerged only recently. It is now understood that PKP3 protein is a component of intercellular junctions, where it affects cell-cell adhesion through interaction with the desmosomal cadherins (14–16, 24, 25). However, the presence of other p120ctn/plakophilin subfamily proteins in cell nuclei or cytoplasm as well as intercellular junctions implies multiple functions for these proteins in different cellular compartments. Some p120CTN/plakophilins are known to be expressed as cytoplasmic and/or nuclear proteins and be translocated to desmosomes during certain stages of differentiation (13, 24–26).

Figure 3. Growth, invasion, and transformation effects of PKP3. (A), representative immunocytochemical images of PKP3-stable transfectants of COS-7 cells stained with anti-c-myc antibody (×200). Exogenous PKP3 protein was detected mainly in the cytoplasm and/or plasma membrane (left and right). (B), morphology of PKP3-transfected cells. (C), expression of exogenous PKP3 protein in stable transfectants, detected by Western blot analysis using anti-c-myc or anti-PKP3 antibody (top), and effect of PKP3 on growth of COS-7 cells (bottom). Stable transfectants expressing high levels of exogenous PKP3 (COS-7-PKP3-1 and COS-7-PKP3-2) and controls (COS-7-mock-1 and COS-7-mock-2) were each cultured in triplicate. At each time point, cell viability was evaluated by the MTT assay. (D), Matrigel invasion assay demonstrating the increased invasive ability of COS-7 cells transfected with human PKP3 expression plasmids. Left, Giemsa staining (×200); right, numbers of cells invading through Matrigel-coated filters. These assays were done three times and in triplicate wells.
For example, dominant cytoplasmic localization of abundant p120ctn/plakophilin subfamily proteins, including PKP3, was detected in some cancer tissues (16, 27, 28). The data in the present study should make clearer the role of the native or the transfected PKP3 protein in the living cells. First, immuno-histochemical and immunocytochemical analyses (Fig. 1C and Fig. 3A) revealed that most of the lung tumors, or PKP3-stable COS-7 cells whose parental cells do not express endogenous PKP3 (14), as confirmed by our RT-PCR analysis, mainly exhibited a cytoplasmic distribution of abundant PKP3, consistent with the result in a previous report (16). Second, semiquantitative RT-PCR analysis revealed that there was no significant correlation of gene expression between PKP3 and PKP3-associated desmosomal components in the lung cancer samples and COS-7 cells. Our data by immunostaining in PKP3-overexpressing cancer cells indicated that the majority of overexpressed PKP3 protein was present as a soluble form mainly at cytoplasm and a small fraction was present as an insoluble form at other compartment like desmosomes. It is well known that β-catenin (CTNNB1), a member of other ARM-protein family associates with the cytoplasmic domain of cadherin and α-catenin (CTNNA) and physiologically functions as an integral component of adherens junctions but accumulates in cytoplasm due to abrogation of its degradation pathway by the extracellular Wnt activation of the Frizzled receptor or selected mutations in CTNNB1/APC/AXIN (17, 29). Accumulated CTNNB1 interacts with Tcf/Lef, thereby transactivating target genes of the transcriptional complex and ultimately leading to cancer in certain type of cells (17, 29). On that basis, we speculate that the imbalance of overexpressed PKP3 levels between its membrane-associated form and the cytoplasmic pool might affect its complex formation with unknown soluble protein(s); one of possible candidates could be DNM1L (see below). The fact that PKP3 is an Arm-related protein and is activated in lung cancer samples and that PKP3-stable COS-7 clones represent transformed cellular phenotype (Fig. 3) doubtlessly suggests that in addition to an important role for cell-

![Figure 4. Identification of DNM1L as a protein interacting with PKP3 in NSCLC.](image)

A, semiquantitative RT-PCR analysis of PKP3, desmosomal proteins known to be associated with PKP3, and DNM1L in NSCLC cell lines. B, reciprocal communoprecipitation of endogenous PKP3 and DNM1L from extracts of lung cancer cell line LC319. Top, Western blot analysis of cell extracts immunoprecipitated with anti-PKP3 antibodies, with endogenous DNM1L protein detected in the immunoprecipitate. Bottom, Western blot of extracts immunoprecipitated with anti-DNM1L antibodies, with endogenous PKP3 protein detected in the immunoprecipitate. IP, immunoprecipitation; IB, immunoblot.
cell adhesion and structures of epithelia in normal cells (13–16), this protein also has an oncogenic function under activating conditions in cytoplasm.

Our treatment of NSCLC cells with specific siRNA to reduce expression of PKP3 protein suppressed growth significantly. On the other hand, cell proliferation was enhanced in cultures of COS-7 cells made to stably express PKP3; those transfectants also revealed a multilayer growth pattern and formed larger colonies than normal COS-7 cells. Introduction of PKP3 also resulted in the formation of many protrusions and promoted the invasiveness in Matrigel. We also documented interaction of endogenous PKP3 with DNM1L, which was overexpressed in NSCLC cells. Finally, clinicopathologic evidence obtained through our tissue microarray experiments showed that lung adenocarcinoma patients with tumors expressing a high level of PKP3 showed shorter cancer-specific survival periods than those with negative or low level of PKP3 expression. Combined with the in vitro data, it is likely that overexpressed PKP3 could be an indicator for a highly malignant phenotype of lung cancer cells.

Dynamin family member proteins containing a GTPase domain, a pleckstrin homology motif and a proline-rich tail participate in receptor-mediated endocytosis at the plasma membrane in organisms ranging from insects to vertebrates (30, 31). Dynamins are known to interact with or work synergistically with a variety of cellular proteins, including the actin cytoskeleton to regulate actin reorganization and subsequently influence to cell shape during cell migration, endocytosis, and secretion (32). On the other hand, dynamin-related GTPases, such as the yeast protein Vps1p highly homologous to human DNM1L, which lack both the pleckstrin homology motif and the proline-rich region, also participate in vesicular transport within the secretory pathway, and are essential for the maintenance of mitochondrial morphology in lower eukaryotes (30, 31). Interestingly, recent reports have indicated that dynamin is a central modulator of protrusive events and is associated to podosome and invadopodia structure (33, 34). Podosomes are expressed in cells of the monocytic lineage, and many studies indicated a significant role of podosomes in adhesion/motility. Invadopodia are prominent in certain aggressive cancer cells or transformed cells and seem directly responsible for focal extracellular matrix degradation (33, 34). To our knowledge, our data is the first evidence of cognate interaction of a desmosomal protein (PKP3) and dynamin family protein (DNM1L), both of which were activated in NSCLC cells.

The combined results strongly suggested that PKP3 is likely to function as an oncogene in NSCLC through interaction with some cytoplasmic protein(s) except for the known PKP3-interacting desmosomal proteins, and to be associated with invasiveness and the highly malignant phenotype of those tumors as shown here by in vitro and in vivo assays. Our evidence indicates that PKP3 is a likely target for development of agents for diagnosis and treatment of NSCLC. Although the precise molecular mechanism underlying our observations is unknown, one possibility is that up-regulation of PKP3 promotes tumor progression, cell motility, and invasion by associating with

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Association of PKP3 overexpression with poor clinical outcomes among adenocarcinoma (ADC) patients. A, immunohistochemical evaluation of PKP3 expression on tissue microarrays (<x100). Examples are shown of strong, weak, and absent PKP3 expression in lung adenocarcinomas and of no expression in normal lung. B, summary of the relationship between high PKP3 expression and clinicopathologic factors in adenocarcinomas. C, Kaplan-Meier analysis of tumor-specific survival in patients with adenocarcinoma according to PKP3 expression (P = 0.009, log-rank test).
DNM1L, activating some signaling pathway(s), or dysregulating cadherin-mediated adhesion. Given the β-catenin-signaling model and what is known about other p120ctn/plakophilins in mammalian cells, it is entirely possible that PKP3 could be involved in signal transduction pathway(s) in cancer cells. It is shown that DNM1L interacts with the GSK-3β through its COOH-terminal region, implying that the PKP3-DNM1L complex may also be involved in cell signaling (35). Elucidation of the mechanism implied by these observations should reveal important new information about cell-cell communication, differentiation, and cancer progression.

In summary, we have shown that overexpressed PKP3 is likely to be an essential contributor to a growth-promoting pathway and to aggressive features of NSCLCs. The data reported here strongly imply the possibility of designing biomarkers and anticancer drugs specific for lung cancer based on the PKP3 molecule. Specific siRNAs should be one of the options to investigate for interfering with this pathway.

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Chiyuki Furukawa, Yataro Daigo, Nobuhisa Ishikawa, et al.


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