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ORIGINAL ARTICLE

TOPK/PBK promotes cell migration via modulation of the PI3K/PTEN/AKT pathway and is associated with poor prognosis in lung cancer

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We integrated four gene expression profile data sets, namely two different pair-matched stage I lung adenocarcinoma data sets, secondary metastatic tumors vs benign tumors and lung tumor metastasizes to the brain, and we identified one kinase, T-LAK Cell-Originated Protein Kinase (TOPK), as a putative gene that promotes metastasis. To delineate the role of TOPK in lung cancer, we showed that overexpression of TOPK, but not a catalytically inactive form of TOPK, can enhance the migration and invasion of lung fibroblasts or cells with low TOPK expression. In addition, TOPK-induced cell migration was shown to be a PI3K/AKT-dependent event. TOPK concurrently promoted AKT phosphorylation at Ser^{473} and decreased the phosphatase and tensin homolog (PTEN) levels, whereas TOPK knockdown had the reverse effects. LY294002, a PI3K inhibitor, did not inhibit the TOPK-induced decrease in PTEN, and co-expression of PTEN significantly reduced TOPK-induced AKT phosphorylation in a dose-dependent manner; these results indicate that the TOPK-mediated PTEN decrease has an upstream role in regulating PI3K/AKT-stimulated migration. Using immunohistochemical analysis of lung cancer tissue samples, we showed that a high TOPK expression level correlates strongly with reduced overall and disease-free survivals. Moreover, an inverse correlation between TOPK and PTEN expression was present and is consistent with the biochemical findings. Finally, a combination of high TOPK and low PTEN expression was inversely correlated with overall and disease-free survivals, independent of other pathologic staging factors. Our results suggest that TOPK is a potential therapeutic target in lung cancer that promotes cell migration by modulating a PI3K/PTEN/AKT-dependent signaling pathway; they also suggest that high TOPK expression, either alone or in combination with a low level of PTEN, may serve as a prognostic marker for lung cancer.

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Introduction

Non-small cell lung cancer (NSCLC) accounts for >80% of cases of lung cancer, which ranks among the most deadly cancers worldwide. Surgical resection remains the mainstay of treatment and provides the best chance for survival. However, the treatment outcome of surgery alone is unsatisfactory; the reported failure rate in stage I NSCLC ranges from 27 to 38%, and ~90% of cancer deaths are associated with tumor recurrence or metastasis (al-Kattan *et al*., 1997; Sugimura *et al*., 2007; Hung *et al*., 2009). To improve survival, a recent meta-analysis suggests the addition of adjuvant chemotherapy after surgery for patients with operable NSCLC (Arriagada *et al*., 2010). However, whether stage I NSCLC patients can benefit from adjuvant chemotherapy remains controversial (Arriagada *et al*., 2004; Strauss *et al*., 2008). The identification of prognostic markers to stratify patients into high- and low-risk groups, with respect to disease-free and overall survivals, is ongoing.

Given that the basic genomic program of the primary tumor may predetermine its metastatic capability, careful examining the expression of putative metastasis-associated genes in the primary tumor may help to identify subclinical micrometastases, predict the risk of recurrent disease, and aid the selection of patients who might benefit from adjuvant chemotherapy. Recently, we set up an integrated microarray analysis to identify novel regulators that participate in metastasis and that might serve as molecular markers to predict relapse after treatment. After integrating four gene expression profile data sets, we focused on one putative metastasis-promoting kinase, T-LAK Cell-Originated Protein Kinase (TOPK), and aimed to decipher its role in lung cancer and cancer metastasis.

TOPK, also known as *PBK* (*P*DZ-*b*inding *k*inase), was previously shown to be overexpressed in activated T-LAK cells and to interact with hDlg through TOPK's

C-terminal PDZ-binding motif (Abe *et al*., 2000; Gaudet *et al*., 2000). TOPK is a 322 amino-acid MAPKK-like serine/threonine kinase and is highly expressed in various types of cancer, such as lymphoma, leukemia, breast cancer, melanoma and colorectal cancers, but is undetectable in normal tissues except the germ cells of testis and several fetal tissues (Abe *et al*., 2000; Simons-Evelyn *et al*., 2001; Nandi *et al*., 2004; Park *et al*., 2006; Zykova *et al*., 2006; Zhu *et al*., 2007). In cultured cells, the expression of TOPK is upregulated during mitosis, when it is thought to be phosphorylated on Thr^{9} and activated by cyclin B1/cdk1 (Matsumoto *et al*., 2004). Because it enhances cyclin B1/cdk1-dependent phosphorylation of PRC1, TOPK has been hypothesized to have a role in cytokinesis (Abe *et al*., 2007; Chen *et al*., 2009b). In correlation with its overexpression in highly proliferative cells and cancer tissues, several lines of evidence have indicated the oncogenic role of TOPK in a panel of cancer cells. For example, knockdown the expression of TOPK by siRNA inhibits the growth and clonogenicity of breast cancer cells, possibly by affecting histone H3-promoted mitotic events (Park *et al*., 2006) or p38-mediated signaling (Ayllon and O'Connor, 2007). TOPK has been shown to mediate UVB-induced JNK activation and augment H-Ras-induced cell transformation (Oh *et al*., 2007) and has a role in DNA damage sensing and repair. Moreover, TOPK serves as an oncogenic MEK that exerts positive feedback on ERK2 to promote colorectal cancer formation *in vitro* and *in vivo* (Zhu *et al*., 2007). Recently, TOPK has been identified as a downstream target of the EWS-FLI chimeric fusion protein, which has a significant role in Ewing sarcoma biology (Herrero-Martin *et al*., 2009). Furthermore, TOPK physically interacts with p53 and may contribute to tumorigenesis through the suppression of p53 function (Nandi *et al*., 2007; Hu *et al*., 2010).

Although accumulating studies have demonstrated the crucial roles of TOPK in cell-cycle regulation and tumorigenesis, its role in lung cancer and cancer metastasis has never been addressed. Here, we report overexpression of TOPK in the majority of lung cancer tissues and cell lines. Ectopic expression of TOPK could promote cell migration and invasion rather than affecting cell proliferation. In addition to the roles proposed in previous studies, TOPK may activate a PI3K/AKT-dependent cell migration through modulating the protein level of phosphatase and tensin homolog (PTEN), which is a negative regulator of PI3K/AKT-dependent signaling. Immunohistochemical (IHC) analysis indicates that high TOPK and/or low PTEN expression levels correlate strongly with reduced overall and disease-free survivals. Overall, our study identifies a novel role for TOPK in regulating PI3K/PTEN/AKT signaling and cell migration/invasion. Given that TOPK is significantly upregulated in metastatic cancers and could promote cell migration and/or invasion, these findings may have implications for TOPK as a promising molecular target for therapy and a prognostic marker for highly recurrent lung cancer.

Results

Identification of TOPK as an upregulated and metastasis-related protein kinase in lung adenocarcinoma via the integration of gene expression data sets

To rapidly prioritize targets for lung cancer therapy, particularly poorly characterized protein kinases involved in metastasis, we set up a bioinformatics platform integrating four transcriptome data sets for the identification of such genes. As a result of our bioinformatic survey, we focused on the poorly characterized gene *TOPK*. First, we used a training microarray data set, namely samples from 27 patients with lung adenocarcinoma confirmed by histopathology, which were subjected to Affymetrix microarray profiling using HG-U133A (Su *et al*., 2007). Of many differentially expressed transcripts between the adjacent normal area and the tumor examined by Wilcoxon signed rank test ($P<0.01$) and tested using the Benjamini and Hochberg false discovery rate correlation ($P<0.01$), *TOPK* expression was upregulated, as shown by boxplot (Figure 1a, left). Two well-known oncogenic protein kinases, *EGFR* and *AURKA*, were shown to be overexpressed in the same data sets (Figure 1a, left), consistent with the idea that microarray profiling can represent an unprecedented platform for discovery novel regulators involved in the carcinogenesis of, for example, lung cancer. Second, to validate this observation, we conducted an independent microarray profiling 25 pair-matched, stage 1 lung adenocarcinoma patient specimens by using a new Affymetrix microarray (HG-U133 plus 2.0, Affymetrix, Santa Clara, CA, USA) chip and the same statistical analyses. Again, *TOPK* expression was upregulated in the tumor compared with the adjacent normal area (Figure 1a, right).

Third, to provide additional information in our *in silico* screening, we created two sets of metastasis signatures. Public accessible microarray data (downloaded from Gene Expression Omnibus) were used to acquire different microarray data sets, which were normalized by Quantile Normalization using R to set up a potential metastatic biomarker identification platform. We compared 225 secondary metastatic tumors (including 20 tumor metastases to the lymph node and 200 metastatic tumors) and 30 benign tumors to obtain sets of potential promoters of metastasis, as described previously (Chen *et al*., 2009a). *TOPK* exhibits differential expression patterns, as shown by boxplot (Figure 1b, left). Finally, because lung cancer often metastasizes to the brain after gefitinib treatment (Omuro *et al*., 2005), we compared six commercially available normal brains and five specimens of lung adenocarcinoma metastasis to brain. Differentially expressed genes were considered to have the potential to participate in lung adenocarcinoma metastasis, and *TOPK* was one of them (Figure 1b, right). The combination of these four distinct microarray data sets identifies *TOPK* as an overexpressed and metastasis-related protein kinase in lung adenocarcinoma. These data raise the possibility that upregulation of *TOPK* might lead to some of the abnormalities found in human lung adenocarcinoma.

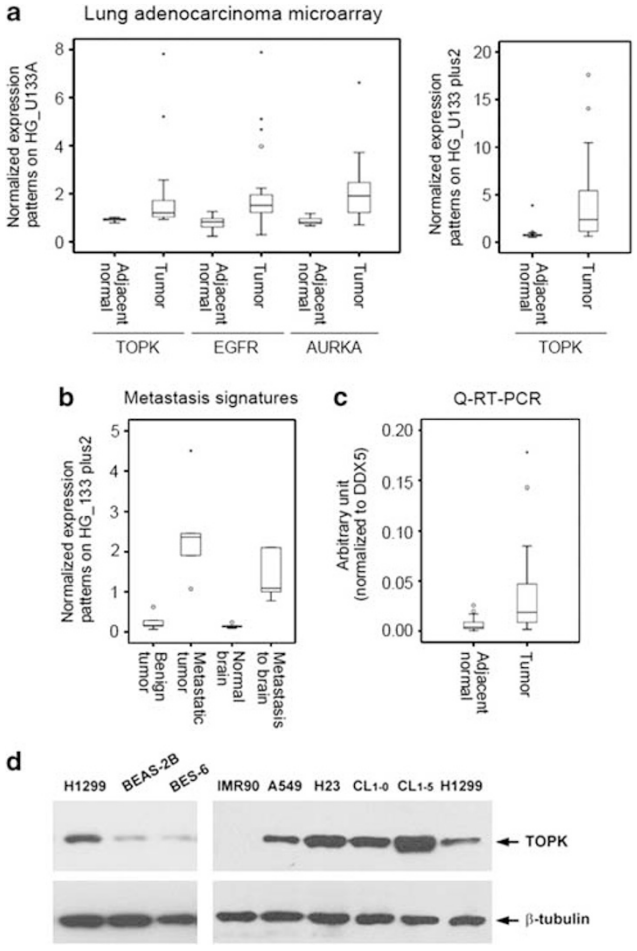

Figure 1 TOPK expression is upregulated in lung adenocarcinomas. (**a**) The microarray expression patterns of *TOPK* (219148_at), *EGFR* (201984_s_at) and *AURKA* (208079_s_at) from 27 lung adenocarcinoma patients were normalized against the expression patterns on HG-U133A chips (left panel). A different set of 25 lung adenocarcinoma patient specimens was subjected to microarray analysis using HG-U133 plus 2.0 chips (right panel). The boxplot shows the data distribution as a grouping classification and indicates that there are statistically significant differences ($P<0.0001$, left panel; $P<0.001$, right panel) between the tumor tissues and the adjacent non-tumor tissues. (**b**) The microarray expression patterns of *TOPK* were compared among 30 benign tumors and 220 metastatic tumors (first two panels) and are shown in a boxplot ($P<0.001$). Differential expression of *TOPK* in five commercially available brain tissues and six brain metastases derived from lung adenocarcinomas is also illustrated ($P=0.043$). (**c**) The mRNA levels of *TOPK* in samples from 24 lung adenocarcinoma patients, which are the same as those in (**a**, left panel), were determined by Q-RT–PCR. The results were normalized to the level of *DDX5* mRNA in each paired sample and plotted in a boxplot ($P=0.004$). (**d**) BEAS-2B, BES-6, IMR90 cells and a panel of lung cancer cell lines were harvested and subjected to immunoblot analysis for detection of TOPK expression. β-Tubulin staining was included as a loading control (BEAS-2B and BES-6: human bronchial epithelial cell line; IMR90: human lung fibroblast cell line; A549, H23, CL_{1-0} and CL_{1-5}: human lung adenocarcinoma cell line; H1299: human lung epithelial carcinoma cell line).

Elevated TOPK expression in lung adenocarcinomas and invasive lung cancer cell lines

To clarify the underlying molecular mechanisms of *TOPK* in lung cancer progression, we first validated the gene expression profiling data for *TOPK*. Quantitative real-time PCR (Q-RT–PCR) was performed on 24 pair-matched lung adenocarcinoma tumors and adjacent non-tumor samples. Overexpression of *TOPK* was observed in 20 out of 24 lung patient tumors, showing a higher signal after normalization to *DDX5*, a novel internal control for lung adenocarcinoma (Su *et al*., 2007), for equal template loading. The average expression level of *TOPK* in lung adenocarcinoma was ~5-fold higher than that in adjacent non-tumor lung tissue samples, as analyzed by boxplot (Figure 1c). Next, we examined and compared the protein expression levels of TOPK in the human lung fibroblast line IMR90 and a panel of lung cancer cell lines. As shown in Figure 1d, TOPK protein was markedly expressed in the lung cancer cell lines and barely detected in BEAS-2B, BES-6 and IMR90 cells. Moreover, the protein level of TOPK was higher in the highly invasive CL_{1-5} as compared with the less invasive CL_{1-0} cells.

Overexpression of TOPK enhances cell migration and invasion in lung cancer cell lines

Because the expression of TOPK correlates with the invasiveness of lung cancer cells, it is likely that the expression of TOPK has a role in cancer metastasis. We, therefore, investigated whether overexpression of TOPK could affect cell migration or invasion in NSCLC cell lines with low levels of endogenous TOPK, such as CL_{1-0} and H1299 cells. We transiently transfected vector control and Flag-tagged TOPK expression plasmids into CL_{1-0} and H1299 cells, respectively, and tested the effect of TOPK on cell migration, invasion and cell proliferation. As expected, exogenous Flag-tagged TOPK was overexpressed in CL_{1-0} and H1299 cells, which resulted in 3.1-fold (CL_{1-0}) and 2.8-fold (H1299) increase in cellular migration and an ~2-fold (CL_{1-0} and H1299) increase in cellular invasion as compared with the control cells (Figures 2a and b). The observed migration- and invasion-promoting abilities of TOPK were largely depended on its kinase activity, because overexpression of a catalytically inactive TOPK (TOPK-kinase-dead (KD)) had no significant effect on cell migration or invasion. In addition, though TOPK is reported to be overexpressed in highly proliferative cells (Simons-Evelyn *et al*., 2001), ectopically overexpression of TOPK neither significantly affect cell proliferation as analyzed by cell counting (Supplementary Figure S1) and MTT assay at 24 h nor affect cell-cycle distribution (data not shown), indicating TOPK could enhance the migratory and invasive ability of lung cancer cells without promoting proliferation.

PI3K/AKT-dependent signaling is critical for TOPK-induced cell migration

Tumor cells possess a broad spectrum of migration and invasion mechanisms that are associated with enhanced metastasis (Friedl and Wolf, 2003). To explore which signal transduction pathways might participate in TOPK-mediated cell migration in lung cancer cells, we investigated three well-defined signaling pathways,

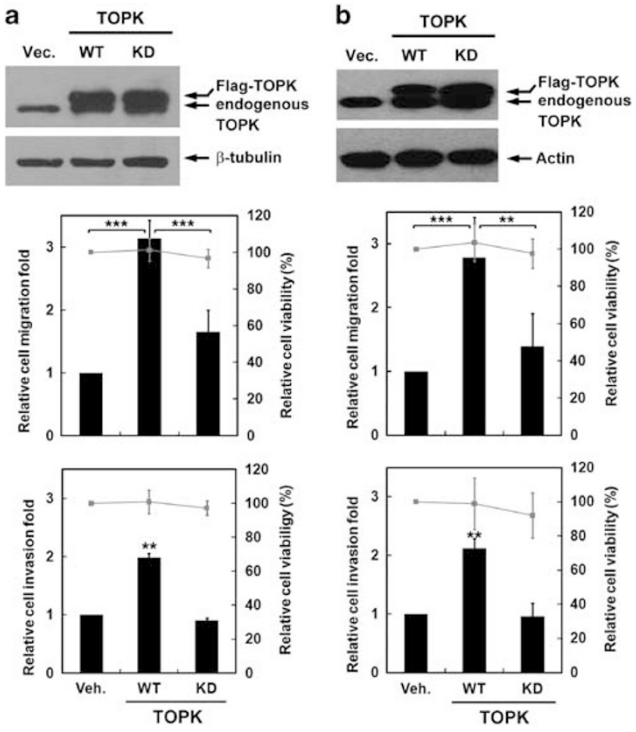

Figure 2 Overexpression of TOPK increases the migration and invasion of lung cancer cell lines. (**a**) CL_{1-0} or (**b**) H1299 cells were transiently transfected with (vehicle; Veh.) or pCMV2-Flag-TOPK (WT) or pCMV2-Flag-TOPK (KD) plasmids. Twenty-two hours after transfection, one portion of the cells was harvested for detection of TOPK and β-tubulin or actin by immunoblotting (upper panels). The other portions of cells were seeded onto Matrigel-coated or uncoated transwell inserts or 96-well plate and incubated for another 24 h. Cell growth was quantified by an MTT assay. The numbers of migrated (middle panels) and invaded (lower panels) cells were then counted and expressed as relative migration or invasion fold as compared with vehicle control ($n = 3$–6) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

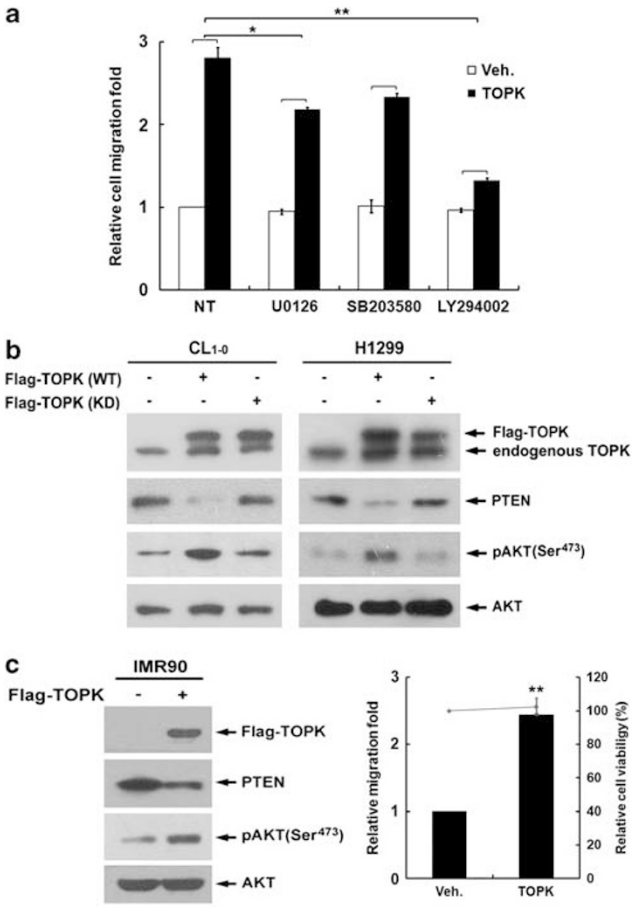

Figure 3 TOPK modulates PTEN/PI3K/AKT signaling in lung cancer cells and lung fibroblasts. (**a**) CL_{1-0} cells were transiently transfected with a vector control or pCMV2-Flag-TOPK plasmid. Twenty-two hours after transfection, cells were seeded onto transwell inserts and incubated for another 24 h. Before counting the migrated cells, three different pharmacological inhibitors, U0126, SB203580 and LY294002 (10 μM), were added into separate transwell inserts, which were then incubated for another 2 h. Then, the number of migrated cells was counted and expressed as cell migration relative to vector transfected and untreated cells (Veh./NT). CL_{1-0} (**b**, left), H1299 (**b**, right) and IMR90 (**c**) cells were transiently transfected with a vector control or pCMV2-Flag-TOPK (WT) or pCMV2-Flag-TOPK (KD) plasmid. Twenty-two hours after transfection, a fraction of the cells was harvested for detection of phospho-AKT (Ser^{473}), AKT, PTEN and TOPK by immunoblotting. The migration and cell viability of TOPK-overexpressing IMR90 cells were also evaluated as described previously (**c**, right) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

namely the MEK/ERK, p38 and PI3K/AKT pathways, using specific pharmacological inhibitors. The increase in cell migration stimulated ectopic expression of TOPK in CL_{1-0} cells was strongly decreased by treatment with LY294002 and inhibited to a smaller extent by U0126 and SB203580 (Figure 3a). Moreover, elevated AKT-Ser^{473} phosphorylation was observed in TOPK-WT-expressing CL_{1-0} cells compared with vehicle and TOPK-KD-expressing cells. Similar results were obtained in H1299 cells (Figure 3b). Together, these data indicate that the PI3K/AKT pathway is involved in TOPK-induced cell migration.

Inverse correlation of AKT phosphorylation and PTEN level in TOPK-overexpressing cells

PTEN is a lipid phosphatase that acts as a tumor suppressor by negatively controlling the PI3K/AKT signaling pathway. Inactivation of PTEN often results in increased AKT activity in many types of tumors, including lung adenocarcinoma (Soria *et al*., 2002; Vivanco and Sawyers, 2002). The ectopic expression of PTEN in various tumor cell lines exerts inhibitory effects on several known biological actions of PI3K signaling pathway, for example, migration (Akca *et al*., 2011). In addressing how TOPK modulates the PI3K/AKT pathway, we noticed that the protein expression level of PTEN was decreased in TOPK-WT-overexpressing cells as compared with vehicle and TOPK-KD-transfected cells (Figure 3b). To provide additional evidence to support this notion, ectopic expression of TOPK in the human lung fibroblast line IMR90, which does not contain detectable endogenous TOPK, was also evaluated. Despite low transfection efficiency in IMR90, overexpression of TOPK resulted in down-regulation of PTEN, upregulation of AKT-Ser^{473}

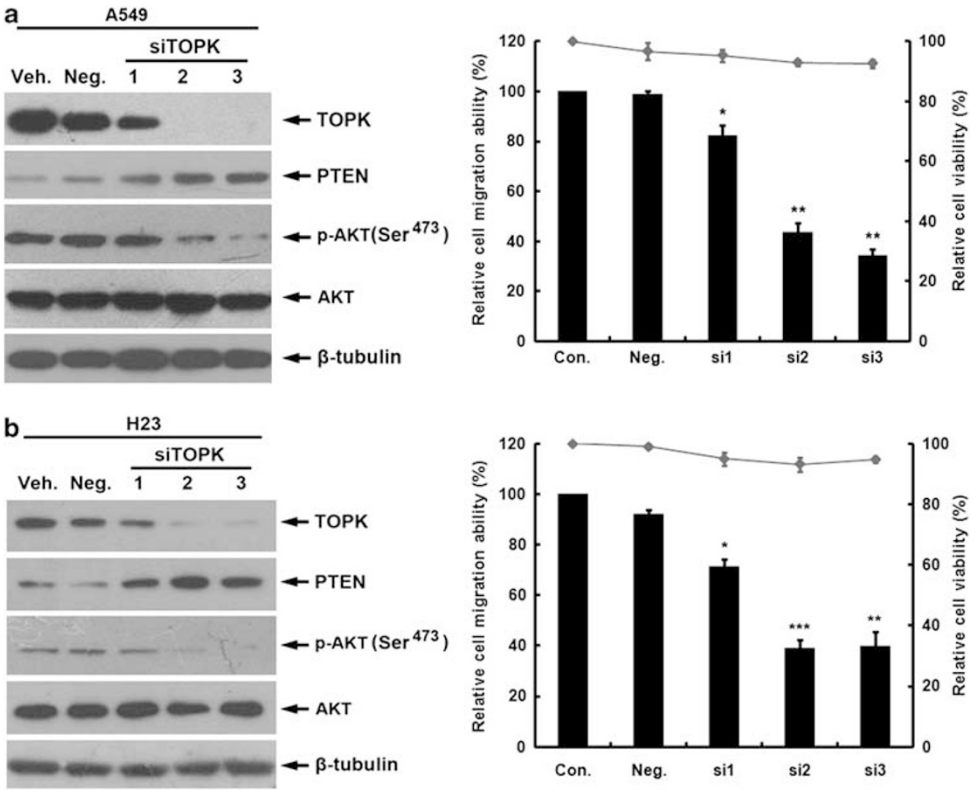

Figure 4 Knockdown of TOPK enhances the expression of PTEN and decreases the activation of AKT in lung cancer cell lines. A549 (**a**) and H23 (**b**) cells were transfected without (vehicle; Veh.) or with a scrambled siRNA (Neg.) or one of three TOPK-specific siRNAs (siTOPK). Forty-eight hours after transfection, a fraction of the cells was harvested for detection of TOPK, PTEN, phospho-AKT (Ser^{473}), AKT and β-tubulin by immunoblotting. A portion of the cells was seeded onto a transwell insert and incubated for another 24 h. The number of migrated cells was then counted (**a**, **b**, right panels) ($*P<0.05$, $**P<0.01$, $***P<0.001$).

phosphorylation and enhanced cell migration (Figure 3c), consistent with our other observations.

siRNA-mediated depletion of endogenous TOPK suppresses lung cancer cell migration and PI3K/AKT signaling

To further confirm that TOPK affects cell migration by modulating the PI3K/PTEN/AKT signaling pathway and rule out the possibility of the off target effects emanating from LY294002, we next examined if TOPK-specific siRNAs could affect cell migration and activation of endogenous AKT in A549 cells. We transfected three chemically synthesized siRNAs specific for TOPK (siTOPK1 ~ 3) and a scrambled control siRNA (Neg.) into A549 cells and analyzed the expression of TOPK at 48 h after transfection. The data showed different degrees of reduction in the TOPK protein in TOPK siRNA-transfected cells, which correlated with decreases in cell migration (Figure 4a). In accordance with the previous results, the reduction of migration in TOPK-depleted cells did not result from differences in cell proliferation rates, as shown in a cell proliferation assay. In addition, the data indicated that AKT-Ser^{473} phosphorylation was greatly reduced in TOPK-depleted cells as compared with that seen in vehicle-treated or scrambled control cells and that this reduction occurred in parallel with an increase in the PTEN protein level (Figure 4a, left). Similar results were also observed in H23 cells (Figure 4b). Together, these results strengthen of the evidence for TOPK modulation of the PI3K/PTEN/AKT signaling pathway.

TOPK induces PI3K/AKT-dependent cell migration by relieving PTEN-mediated negative regulation

To determine whether TOPK mediated AKT-dependent cell motility by regulating the PTEN protein level, we first examined whether co-treating cells with LY294002, a PI3K inhibitor, could affect the TOPK-induced decrease in PTEN. As shown in Figure 5a, the presence of LY294002 significantly inhibited the phosphorylation of AKT but had no effect on the TOPK-induced reduction of PTEN. Moreover, when PTEN was ectopically expressed, TOPK-induced phosphorylation of AKT-Ser^{473} was blocked in a dose-dependent manner (Figure 5b). Because PTEN-G129E, which lacks lipid phosphatase activity, had a smaller effect on TOPK-mediated AKT activation, the data suggest the lipid phosphatase activity of PTEN is crucial for negative regulating AKT activity in lung cancer cells and TOPK that can promote AKT-dependent cell migration by downregulating the protein level of PTEN (Figure 5c).

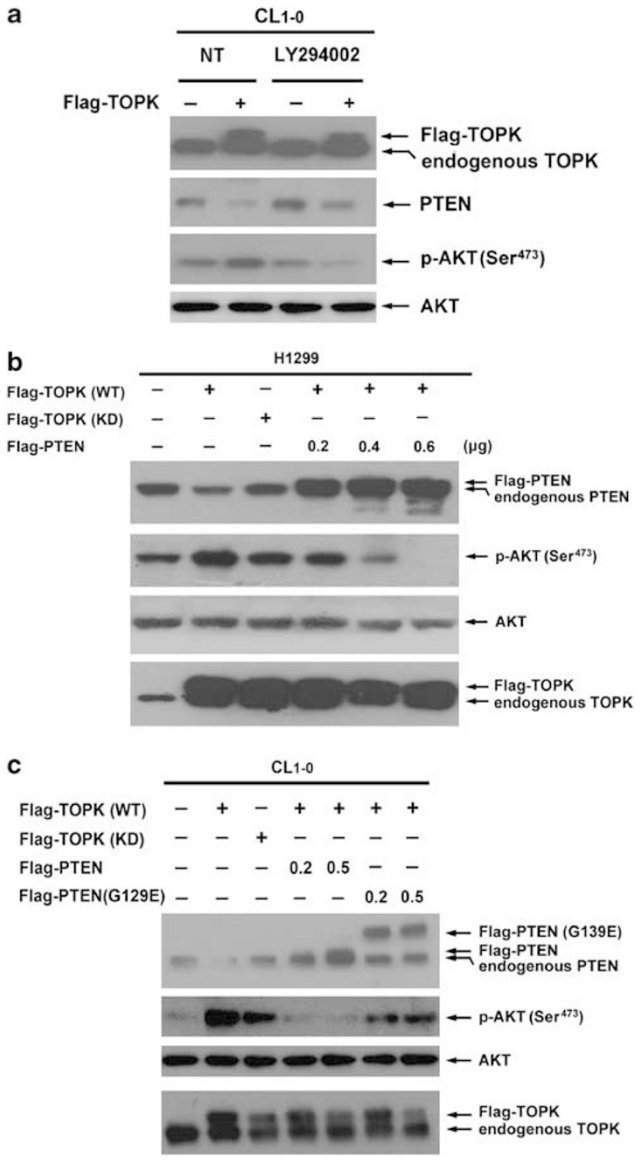

Figure 5 Overexpression of PTEN reverses TOPK-mediated AKT activation. (**a**) One portion of cells from Figure 3a was harvested for detection of phospho-AKT (Ser^{473}), AKT, PTEN and TOPK by immunoblotting. H1299 (**b**) and CL_{1-0} (**c**) cells were transiently transfected with a vector control, pFlag-CMV2-TOPK (WT), pFlag-CMV2-TOPK (KD) and pFlag-CMV2-PTEN or pFlag-CMV2-PTEN (G129E) plasmid in different combination as indicated. Twenty-two hours after transfection, cells were harvested for detection of phospho-AKT (Ser^{473}), AKT, PTEN and TOPK by immunoblotting.

TOPK is a marker for poor prognosis in lung cancer

To provide an independent validation, the prognostic value of TOPK expression was determined by assessing its immunoreactivity using 119 human lung cancer specimens, which were not the same as those examined in our microarray studies (Figure 1), with known clinical follow-up records. High tumor TOPK expression levels (scores of 2 and 3) were more strongly associated with patients with reduced overall and disease-free survivals relative to low TOPK expression level (scores of 0 and 1) (Figure 6). The relationships between the levels of TOPK expression and the clinicopathologic characteristics of lung cancer are summarized in Table 1. Overexpression of TOPK in lung cancer is associated with advanced stage ($P<0.001$) and lymph node metastasis ($P=0.01$). The univariate survival analysis demonstrated that the TOPK score and the pathologic assessment of the primary tumor (T), lymph nodes (N) and metastases (M) had an impact on overall and disease-free survivals (Table 2). The multivariate analysis also showed that the TOPK score and M status significantly affected the overall and disease-free survivals (Table 2). Taken together, our data indicate that a high level of TOPK can be used as an independent prognostic factor in lung cancer.

TOPK expression is inversely associated with PTEN expression in lung cancer patients

To investigate the interplay between TOPK and PTEN in lung cancer patients, we performed IHC analysis of TOPK and PTEN in serial sections of lung cancer tissues. The representative IHC staining for TOPK and PTEN showed a trend toward inverse staining pattern in normal lung, lung adenocarcinoma and lung squamous cell carcinoma (Figure 7). We next investigated the prognostic significance of PTEN by IHC analysis. Supplementary Figure S2 shows that a high PTEN expression level (scores of 2 and 3) correlated strongly with better overall and disease-free survivals relative to low PTEN expression level (scores of 0 and 1). The relationships between PTEN expression and clinicopathologic characteristics of lung cancer are summarized in Supplementary Table S1. The multivariate analysis showed that the PTEN score ($P=0.025$), N status ($P=0.022$) and M status ($P=0.001$) significantly affected overall survival (Supplementary Table S2). The PTEN score, however, strongly but non-significantly affected the disease-free survival ($P=0.086$) as assessed by multivariate analysis. We further investigated the degree of inverse correlation between TOPK and PTEN expression in serial sections of human lung cancer tissues. The IHC analysis of lung cancer specimens revealed an inverse correlation between TOPK and PTEN expression (Figure 8a), with a correlation coefficient $=-0.221$ ($P=0.016$), as analyzed by Spearman's non-parametric correlation test (Supplementary Table S3). Moreover, high TOPK and low PTEN expression, when taken together, were correlated with poor overall ($P<0.001$) and disease-free survivals ($P=0.002$), compared with patients with low TOPK and high PTEN expression (Figures 8b and c).

Discussion

In an attempt to identify genes associated with metastasis that could serve as prognostic makers or therapeutic targets in lung cancer, we have integrated the transcriptomes of lung adenocarcinoma and metastasis signature data sets and identified TOPK as a potential target. Here, by overexpressing TOPK in lung

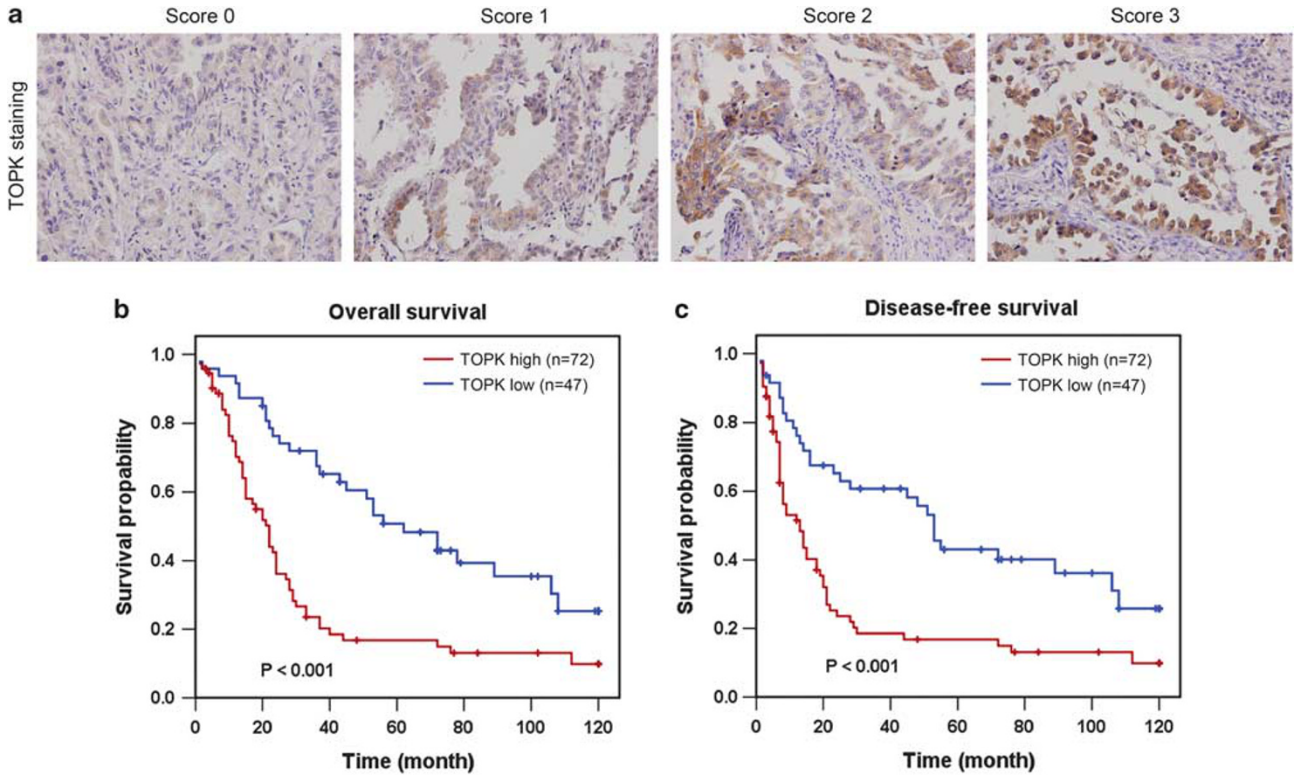

Figure 6 Overexpression of TOPK is a marker of poor prognosis in lung cancer. (**a**) TOPK protein expression in representative lung cancer specimen. TOPK levels were quantified according to the cytoplasmic staining intensity of TOPK. The results were classified into two groups according to the intensity of staining: in the low-expression group, either no staining was present (staining intensity score = 0) or positive staining was detected in $<10\%$ of the cells (staining intensity score = 1); in the high-expression group, positive immunostaining was present in 10–30% (staining intensity score = 2) or $>30\%$ of the cells (staining intensity score = 3). Kaplan–Meier plot of overall survival (**b**) and disease-free survival (**c**) in 119 lung cancer patients, stratified by TOPK expression.

fibroblasts and lung cancer cells with low TOPK expression, we have demonstrated that TOPK can promote cell migration and invasion. Complementary results were obtained in TOPK siRNA-transfected cells. In addition, TOPK may stimulate AKT-dependent cell migration by relieving a PTEN-dependent suppressive effect to facilitate cancer metastasis. Because microarray studies of NSCLC showed an association between patient survival and various gene expression profiles, as recently reviewed (Kratz and Jablons, 2009), we demonstrate that high TOPK expression is a predictor of unfavorable prognosis for lung cancer patients, supporting TOPK as a novel prognostic marker and valuable therapeutic target in lung cancer.

TOPK has been demonstrated to regulate p38MAPK, JNK and ERK/MAPK signaling, possibly in a cell type-dependent manner (Abe *et al*., 2000; Oh *et al*., 2007; Zhu *et al*., 2007). Dysregulation of MAPK signaling highlights the role of TOPK in oncogenesis. In addition, TOPK has been demonstrated to activate p38-mediated cell motility in response to growth factor stimulation (Ayllon and O'Connor, 2007). However, whether TOPK can promote cell migration on its own has never been addressed. Here, we show that overexpression of TOPK enhances cell migration and invasion in lung cancer cells. Though inhibition of p38MAPK and ERK/MAPK slightly inhibited TOPK-induced cell motility, more potent inhibition of cell migration occurred when PI3K signaling was inhibited (Figure 3a). Ectopic expression of TOPK promoted AKT phosphorylation, while decreasing the level of PTEN, but had no effect on ERK or p38MAPK activation (data not shown), indicating that PI3K/AKT-dependent signaling is more crucial for TOPK-promoted cell migration in the lung cancer cells examined.

Because reverse transcription–PCR showed that mRNA levels of PTEN were unchanged in TOPK-transfected cells (data not shown), downregulation of PTEN by TOPK must be a post-transcriptional event. PTEN is regulated by several mechanisms, including phosphorylation and ubiquitination, which govern its stability and activity through its subcellular localization (Chalhoub and Baker, 2009). Phosphorylation of the C-terminal tail stabilizes the PTEN protein but decreases its activity toward PIP_3 by repelling it from the membrane. We found a decrease in endogenous phospho-PTEN (Ser380/Thr382/Thr383) in TOPK-overexpressing cells. However, TOPK can neither phosphorylate nor interact with PTEN (data not shown), suggesting that TOPK-elicited downregulation of PTEN may not be a direct effect. To explore whether the observed decrease in PTEN protein expression levels was attributable to proteasome-mediated protein degradation, we analyzed the protein stability of PTEN in TOPK-overexpressing cells by addition of cyclohexamide in the presence or absence of a proteasome

Table 1 Relationship between TOPK expression and clinicopathologic factors in 119 lung cancer patients

Characteristics	*TOPK expression*		P-*value*[a]
	Low (0,1) (n = *47*)	*High (2,3)* (n = *72*)	
Age			0.64
Years (mean ± s.d.)	62.0 ± 9.5	60.0 ± 10.3	
Sex			0.43
Male	27	36	
Female	20	36	
Smoking status			0.65
No	30	43	
Yes	17	29	
Histological type			
Adenocarcinoma	31	40	0.08
Squamous cell carcinoma	9	29	
Large cell carcinoma	7	3	
Stage[b]			<0.001
I + II	32	18	
III + IV	15	54	
Tumor status			0.07
T1–2	41	53	
T3–4	6	19	
Lymph node status			0.01
N0	25	18	
N1–3	22	43	
Distal metastasis status			0.37
M0	35	48	
M1	12	24	
Recurrence status			0.08
No	30	34	
Yes	17	38	

Abbreviation: TOPK, T-LAK Cell-Originated Protein Kinase.
[a]*P*-value for age was derived from a two-tailed Student's *t*-test; other *P*-values were derived with a two-tailed Pearson's χ^2 test.
[b]The tumor stage, tumor, lymph node and distal metastasis status were classified according to the international system for staging lung cancer.

Table 2 Cox univariate and multivariate regression analysis of TNM prognostic factors and TOPK expression for overall survival (OS) and disease-free survival (DFS) in 119 lung cancer patients

Variables	*Comparison*	*HR (95% CI)*	P-*value*
Cox univariate analysis (OS)			
T	T1–T2; T3–T4	1.832 (1.159–2.894)	0.01*
N	N0; N1–N3	2.037 (1.282–3.237)	0.003*
M	M0; M1	2.628 (1.673–4.129)	<0.001*
TOPK	Low (0,1); high (2,3)	2.501 (1.585–3.946)	<0.001*
Cox multivariate analysis (OS)			
T	T1–T2; T3–T4	1.007 (0.613–1.653)	0.978
N	N0; N1–N3	1.451 (0.894–2.357)	0.132
M	M0; M1	2.446 (1.520–3.935)	<0.001*
TOPK	Low (0,1); high (2,3)	2.323 (1.430–3.775)	0.001*
Cox univariate analysis (DFS)			
T	T1–T2; T3–T4	1.944 (1.229–3.076)	0.004*
N	N0; N1–N3	2.079 (1.305–3.314)	0.002*
M	M0; M1	2.319 (1.479–3.637)	<0.001*
TOPK	Low (0,1); high (2,3)	2.281 (1.446–3.599)	<0.001*
Cox multivariate analysis (DFS)			
T	T1–T2; T3–T4	1.177 (0.723–1.917)	0.512
N	N0; N1–N3	1.532 (0.941–2.495)	0.086
M	M0; M1	2.072 (1.300–3.305)	0.002*
TOPK	Low (0,1); high (2,3)	2.021 (1.252–3.262)	0.004*

Abbreviations: CI, confidence interval; HR, hazards ratio; M, metastases; N, lymph nodes; T, tumor; TOPK, T-LAK Cell-Originated Protein Kinase.
Asterisk represents statistically significant values.

inhibitor, MG132. As expected, the reduction in endogenous PTEN in TOPK-overexpressing cells was rescued when the proteasome activity was blocked (data not shown), indicating that TOPK modulates the protein stability of PTEN by stimulating it to undergo proteasome-dependent degradation. PTEN stability has been found to be regulated by NEDD4-1, which serves as its E3 ubiquitin ligase (Wang *et al*., 2007). Therefore, it is of interest to investigate whether TOPK could regulate NEDD4-1 and thus trigger PTEN degradation.

Because of the crucial roles of TOPK in cell-cycle regulation and in tumorigenesis, its potential application in the clinic has also been evaluated. Recently, Zlobec *et al*. (2010) showed that expression of TOPK, in combination with KRAS and/or BRAF mutations, is linked to an unfavorable prognosis in sporadic colorectal cancer. In this study, we also found that high expression of TOPK is significantly associated with advanced stage and lymph node involvement (Table 1). In addition, high expression of TOPK is a prognostic factor predicting poor outcome for lung cancer patients in terms of both overall and disease-free survivals (Figure 6). Consistent with the current knowledge in the field, distal metastasis (M) and lymph node status (N) were shown to be independent and powerful prognostic factors for overall and disease-free survivals in the Cox regression analysis. Notably, the hazards ratio (HR) of overall and disease-free survivals for TOPK is higher than that of lymph node status (N) and close to that of distal metastasis status (M) (Table 2). The expression of PTEN did not show better predictive value as compared with TOPK in IHC analysis. This may be due to alteration of protein expression as well as the mutation or deletion of PTEN, which could render cells tumorigenic. In addition, we have overexpressed TOPK in PTEN-deficient CL_{1-5} cells, and the results showed that TOPK could still promote cell migration, possibly through a PTEN/AKT-independent mechanism (Supplementary Figure S3).

Subgroup analysis using both TOPK and PTEN expression levels further showed that patients with high TOPK and low PTEN levels had a significantly poorer outcome in terms of overall and disease-free survivals compared with the group of patients with low TOPK/high PTEN levels. Moreover, the 5-year survival probability of the low TOPK/high PTEN group is more than three times greater than that of patients with high TOPK/low PTEN expression (Figure 8). These results indicate that TOPK expression is clinically associated with PTEN expression and can be used as an

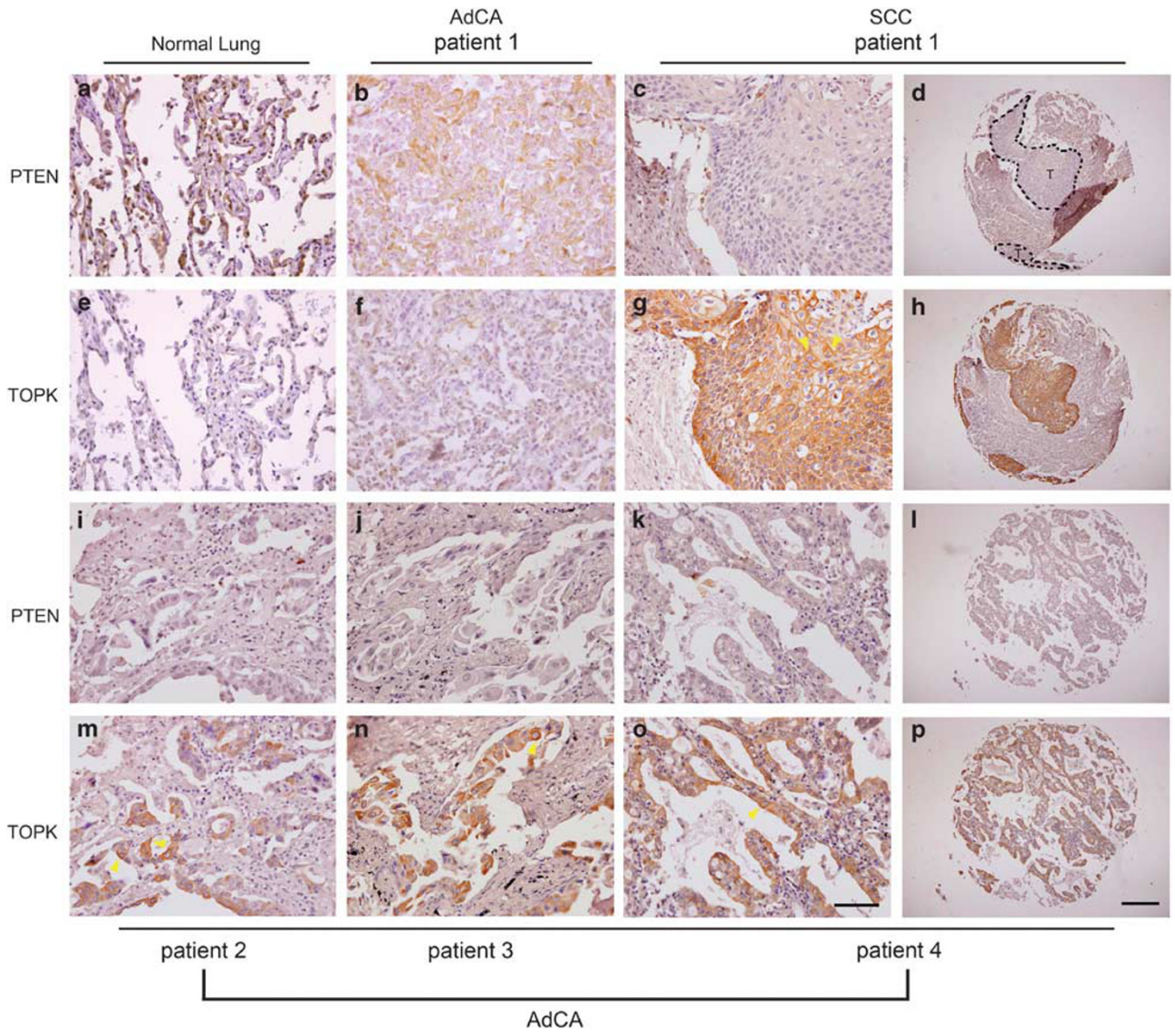

Figure 7 IHC staining of endogenous PTEN and TOPK protein expression in paraffin sections of normal lung tissue and lung cancer samples. Endogenous PTEN expression was detected by immunohistochemistry in normal lung (**a**), adenocarcinomas (AdCAs) (**b**, **i**–**l**) and squamous cell carcinomas (SCCs) (**c**, **d**). Note the strong cytoplasmic expression of PTEN protein found in normal lung and AdCA samples. Also note the weak or absent PTEN protein expression in AdCA samples 2–4 and the SCC samples. Endogenous TOPK expression was detected by immunohistochemistry in AdCA (**m**–**p**) and SCC (**g**, **h**) samples. Note the strong cytoplasmic TOPK staining in AdCA samples 2–4 and SCC sample (**g**). Also note the weak to absent TOPK expression in normal lung (**e**) and AdCA sample 1 (**f**). As indicated, the intensity of TOPK was higher in AdCA and SCC tissue as compared with normal lung; however, the expression level of PTEN was not always low in cancer tissues, such as in AdCA patient 1. We also outlined the edge of tumor with a dashed line in (**d**) in order to allow for comparison with (**h**). Yellow arrowheads indicate strong membrane expression of TOPK in SCC (**g**) and AdCA (**m**, **n**, **p**). The scale bar in (**o**) represents 50 μm. The scale bar in (**p**) represents 200 μm.

independent prognostic factor to predict the treatment outcomes of patients with lung cancer. As this was a retrospective study, patient selection bias and time trend bias were inevitable. Although our results demonstrate that TOPK is a significant prognostic indicator in lung cancer, larger prospective randomized trials are required to further validate the prognostic value of TOPK.

In completely resected NSCLC, disease relapse rates remain high. Adjuvant chemotherapy after complete resection has been shown to improve survival in patients with NSCLC. The requirement for molecular markers to identify, for example, stage II patients with good prognosis who could be spared chemotherapy and high-risk stage 1A patients who might need chemotherapy is an important unmet medical need. The combination of an integrated microarray with empirical analyses suggests that TOPK is a potential therapeutic target and prognostic marker for lung cancer. The question of whether TOPK could further be used for risk stratification in clinical practice, in a similar manner to the application of Oncotype DX in breast cancer screening, warrants further investigation.

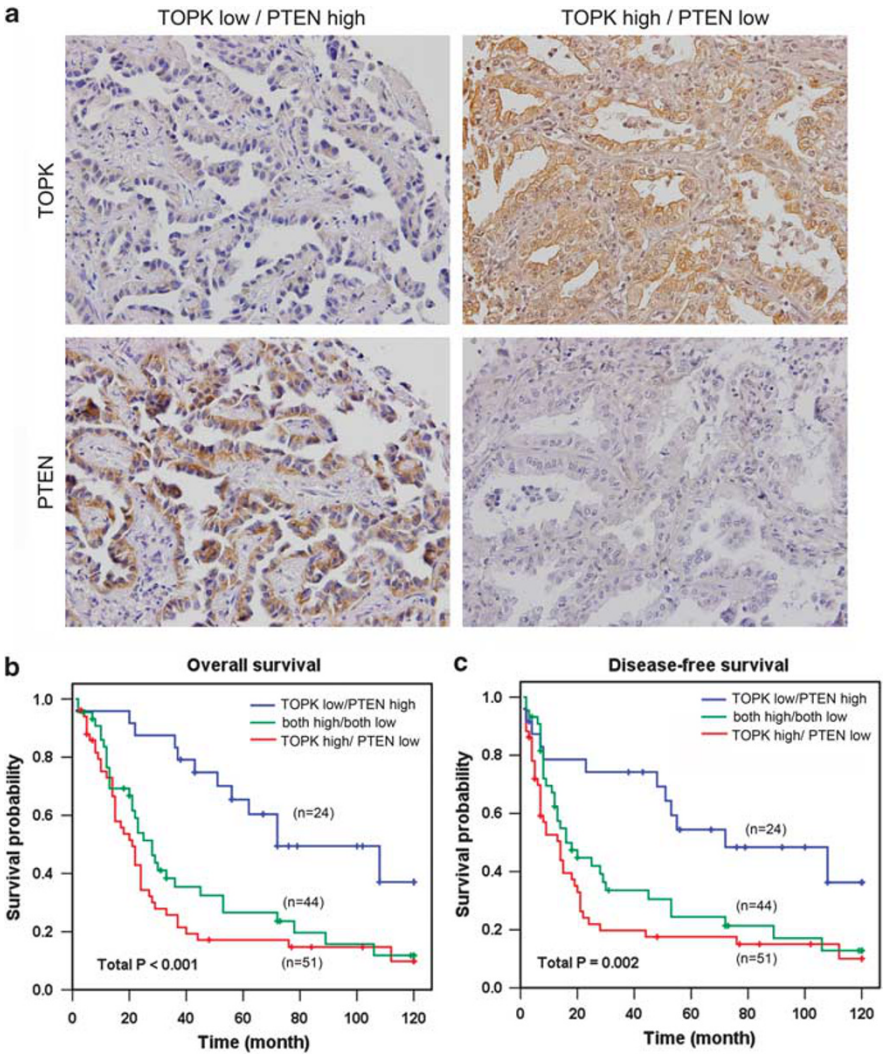

Figure 8 An inverse correlation between TOPK and PTEN expression is a potential prognostic factor in lung cancer. (**a**) IHC staining of TOPK and PTEN in serial sections. Note the inverse correlation between the level of TOPK and that of PTEN. Overall (**b**) and disease-free (**c**) survivals of 119 patients with respect to high TOPK and low PTEN expression.

Materials and methods

Clinical samples for microarray and Q-RT–PCR analysis

Two lung adenocarcinoma microarray data sets were used in this study. First, a total of 66 samples from our previous study were used for microarray analysis via HG-U133A chip (Su *et al*., 2007). Second, 50 additional samples, corresponding to 25 pair-matched surgical samples from lung cancer patients, were subjected to microarray analysis via HG-U133 plus 2.0 chip. These two data sets have been deposited in NCBI's Gene Expression Omnibus and are accessible through Gene Expression Omnibus series accession numbers GSE7670 and GSE27262. Both tumor and adjacent normal tissues were snap frozen in liquid nitrogen, and RNA samples were isolated for microarray analysis and subsequent Q-RT–PCR studies. The mRNA expression level of *TOPK* in 18 pair-matched tumor and adjacent normal samples from lung cancer patients was validated using Q-RT–PCR, which was performed with a Taq-Man probe (Applied Biosystems, Carlsbad, CA, USA). Assays were performed in triplicate using Applied Biosystems Model 7700 instruments (Applied Biosystems). Data are represented as mean ± s.d. To analyze the distribution of tumor and adjacent normal samples, we performed the Wilcoxon signed rank test between two groups for statistical analysis. Additional information is in Supplementary section.

Cells and reagents

All cell culture-related reagents were purchased from Invitrogen (Carlsbad, CA, USA). Human lung cancer cell lines A549, H23 and H1299, as well as IMR90 and BEAS-2B cells were purchased from American Type Culture Collection. Human lung adenocarcinoma cell lines, CL_{1-0} and CL_{1-5}, were kind gifts from Dr Pan-Chyr Yang. All cells were cultured in appropriate medium, for example, RPMI-1640 for A549, CL_{1-0} and CL_{1-5}; DMEM for IMR90, H1299 and H23. Each medium was supplemented with 10% fetal bovine serum, 2 mM of L-glutamine and 1% penicillin/streptomycin. Pharmacological inhibitors used (LY294002, U0126 and SB203580) were from Cell Signaling (Danvers, MA, USA). Primary antibodies (dilutions) used include anti-TOPK (1:1000, BD Biosciences, Bedford, MA, USA), anti-Flag M2 (1:3000; Sigma-Aldrich, St Louis, MO, USA), anti-phospho-AKT (1:1000, Cell Signaling), anti-AKT (1:1000, Cell Signaling), anti-PTEN (1:1000, Cell Signaling), anti-

β-tubulin (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-HA (1:3000, Upstate, Lake Placid, NY, USA).

Plasmids

To construct the TOPK and PTEN expression plasmids, the sequence of TOPK and PTEN cDNA was PCR amplified from the MCG clone using Pfu Turbo DNA polymerase (Stratagene, Santa Clara, CA, USA), respectively. cDNA fragments were then inserted into the *CPO*1 sites of pCMV-2-FLAG expression vector. We also generated a KD mutant, in which Lys64 and Lys65 were substituted with Ala (K64-65A) (Gaudet *et al*., 2000). FLAG-PTEN-G129E expression plasmid was subcloned from pCLXSN-PTEN-G129E plasmid (Furnari *et al*., 1997).

Transfection

For transient transfection, 50% confluent cells were incubated with 1 μg DNA and lipofectamine reagent (ratio 1:6), which were mixed according to the manufacturer's instructions (Invitrogen). To knockdown intrinsic expression of TOPK, cells were transfected with 50 nM of TOPK-specific siRNA. Three siRNA sequences directed against TOPK (siTOPK1: 5′-GC AGCCAUAAUUUUAAAAG dTdT-3′, siTOPK2: 5′-CCCU GAGGCUUGUUACAUU dTdT-3′, siTOPK3: 5′-GCUCU GGAAACAGAUGUCU dTdT-3′) were purchased from Applied Biosystems. A scrambled siRNA duplex was also obtained as a negative control. In general, the transfection efficiency was ~50–70%, except for IMR90 (~30%). At 24 or 48 h after transfection, cells were harvested and subjected to a migration, invasion or proliferation assay and the protein level of TOPK was determined by western blotting as described previously (Chen *et al*., 2009a).

IHC staining

The tissues used were from Kaohsiung Medical University Hospital. All clinical samples were obtained, with informed consent and institutional review board approval, from patients undergoing tumor resection or surgical procedures at Kaohsiung Medical University. Patient information, including gender, age and histopathological diagnoses, was collected. The surgical specimens had been fixed in formalin and embedded in paraffin before they were archived. We used the archived specimens for IHC staining. Follow-up of patients was carried out for up to 200 months. A four-point staining intensity scoring system was devised for determining the relative expression of TOPK in cancer specimens; the staining intensity score ranged from 0 (no expression) to 3 (maximal expression). All of the IHC staining, results were reviewed and scored independently by two pathologists. The antibodies included anti-human TOPK (1:100, Cell Signaling) and PTEN (1:50, Dako, Carpinteria, CA, USA). Immunodetection was performed with an HRP-DAB detection kit (Vector Laboratories, Burlingame, CA, USA).

Statistical analyses

Cox proportional hazards regression was used to test the prognostic significance of factors in univariate and multivariate models. Spearman's rank correlations were determined for comparison of TOPK and PTEN IHC staining. All statistical tests were two-sided, and $P<0.05$ was considered significant ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

Conflict of interest

The authors declare no conflict of interest.

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References

Abe Y, Matsumoto S, Kito K, Ueda N. (2000). Cloning and expression of a novel MAPKK-like protein kinase, lymphokine-activated killer T-cell-originated protein kinase, specifically expressed in the testis and activated lymphoid cells. *J Biol Chem* **275**: 21525–21531.

Abe Y, Takeuchi T, Kagawa-Miki L, Ueda N, Shigemoto K, Yasukawa M *et al*. (2007). A mitotic kinase TOPK enhances Cdk1/cyclin B1-dependent phosphorylation of PRC1 and promotes cytokinesis. *J Mol Biol* **370**: 231–245.

Akca H, Demiray A, Tokgun O, Yokota J. (2011). Invasiveness and anchorage independent growth ability augmented by PTEN inactivation through the PI3K/AKT/NFkB pathway in lung cancer cells. *Lung Cancer* **73**: 302–309.

al-Kattan K, Sepsas E, Fountain SW, Townsend ER. (1997). Disease recurrence after resection for stage I lung cancer. *Eur J Cardiothorac Surg* **12**: 380–384.

Arriagada R, Auperin A, Burdett S, Higgins JP, Johnson DH, Le Chevalier T *et al*. (2010). Adjuvant chemotherapy, with or without postoperative radiotherapy, in operable non-small-cell lung cancer: two meta-analyses of individual patient data. *Lancet* **375**: 1267–1277.

Arriagada R, Bergman B, Dunant A, Le Chevalier T, Pignon JP, Vansteenkiste J. (2004). Cisplatin-based adjuvant chemotherapy in patients with completely resected non-small-cell lung cancer. *N Engl J Med* **350**: 351–360.

Ayllon V, O'Connor R. (2007). PBK/TOPK promotes tumour cell proliferation through p38 MAPK activity and regulation of the DNA damage response. *Oncogene* **26**: 3451–3461.

Chalhoub N, Baker SJ. (2009). PTEN and the PI3-kinase pathway in cancer. *Annu Rev Pathol* **4**: 127–150.

Chen CH, Lai JM, Chou TY, Chen CY, Su LJ, Lee YC *et al*. (2009a). VEGFA upregulates FLJ10540 and modulates migration and invasion of lung cancer via PI3K/AKT pathway. *PLoS ONE* **4**: e5052.

Chen TC, Lee SA, Hong TM, Shih JY, Lai JM, Chiou HY *et al*. (2009b). From midbody protein-protein interaction network construction to novel regulators in cytokinesis. *J Proteome Res* **8**: 4943–4953.

Friedl P, Wolf K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* **3**: 362–374.

Furnari FB, Lin H, Huang HS, Cavenee WK. (1997). Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc Natl Acad Sci USA* **94**: 12479–12484.

Gaudet S, Branton D, Lue RA. (2000). Characterization of PDZ-binding kinase, a mitotic kinase. *Proc Natl Acad Sci USA* **97**: 5167–5172.

Herrero-Martin D, Osuna D, Ordonez JL, Sevillano V, Martins AS, Mackintosh C *et al*. (2009). Stable interference of EWS-FLI1 in an Ewing sarcoma cell line impairs IGF-1/IGF-1R signalling and reveals TOPK as a new target. *Br J Cancer* **101**: 80–90.

Hu F, Gartenhaus RB, Eichberg D, Liu Z, Fang HB, Rapoport AP. (2010). PBK/TOPK interacts with the DBD domain of tumor suppressor p53 and modulates expression of transcriptional targets including p21. *Oncogene* **29**: 5464–5474.

Hung JJ, Hsu WH, Hsieh CC, Huang BS, Huang MH, Liu JS *et al*. (2009). Post-recurrence survival in completely resected stage I non-small cell lung cancer with local recurrence. *Thorax* **64**: 192–196.

Kratz JR, Jablons DM. (2009). Genomic prognostic models in early-stage lung cancer. *Clin Lung Cancer* **10**: 151–157.

Matsumoto S, Abe Y, Fujibuchi T, Takeuchi T, Kito K, Ueda N *et al*. (2004). Characterization of a MAPKK-like protein kinase TOPK. *Biochem Biophys Res Commun* **325**: 997–1004.

Nandi A, Tidwell M, Karp J, Rapoport AP. (2004). Protein expression of PDZ-binding kinase is up-regulated in hematologic malignancies and strongly down-regulated during terminal differentiation of HL-60 leukemic cells. *Blood Cells Mol Dis* **32**: 240–245.

Nandi AK, Ford T, Fleksher D, Neuman B, Rapoport AP. (2007). Attenuation of DNA damage checkpoint by PBK, a novel mitotic kinase, involves protein-protein interaction with tumor suppressor p53. *Biochem Biophys Res Commun* **358**: 181–188.

Oh SM, Zhu F, Cho YY, Lee KW, Kang BS, Kim HG *et al*. (2007). T-lymphokine-activated killer cell-originated protein kinase functions as a positive regulator of c-Jun-NH2-kinase 1 signaling and H-Ras-induced cell transformation. *Cancer Res* **67**: 5186–5194.

Omuro AM, Kris MG, Miller VA, Franceschi E, Shah N, Milton DT *et al*. (2005). High incidence of disease recurrence in the brain and leptomeninges in patients with nonsmall cell lung carcinoma after response to gefitinib. *Cancer* **103**: 2344–2348.

Park JH, Lin ML, Nishidate T, Nakamura Y, Katagiri T. (2006). PDZ-binding kinase/T-LAK cell-originated protein kinase, a putative cancer/testis antigen with an oncogenic activity in breast cancer. *Cancer Res* **66**: 9186–9195.

Simons-Evelyn M, Bailey-Dell K, Toretsky JA, Ross DD, Fenton R, Kalvakolanu D *et al*. (2001). PBK/TOPK is a novel mitotic kinase which is upregulated in Burkitt's lymphoma and other highly proliferative malignant cells. *Blood Cells Mol Dis* **27**: 825–829.

Soria JC, Lee HY, Lee JI, Wang L, Issa JP, Kemp BL *et al*. (2002). Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. *Clin Cancer Res* **8**: 1178–1184.

Strauss GM, Herndon 2nd JE, Maddaus MA, Johnstone DW, Johnson EA, Harpole DH *et al*. (2008). Adjuvant paclitaxel plus carboplatin compared with observation in stage IB non-small-cell lung cancer: CALGB 9633 with the Cancer and Leukemia Group B, Radiation Therapy Oncology Group, and North Central Cancer Treatment Group Study Groups. *J Clin Oncol* **26**: 5043–5051.

Su LJ, Chang CW, Wu YC, Chen KC, Lin CJ, Liang SC *et al*. (2007). Selection of DDX5 as a novel internal control for Q-RT-PCR from microarray data using a block bootstrap re-sampling scheme. *BMC Genomics* **8**: 140.

Sugimura H, Nichols FC, Yang P, Allen MS, Cassivi SD, Deschamps C *et al*. (2007). Survival after recurrent nonsmall-cell lung cancer after complete pulmonary resection. *Ann Thorac Surg* **83**: 409–417; discussion 417–408.

Vivanco I, Sawyers CL. (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* **2**: 489–501.

Wang X, Trotman LC, Koppie T, Alimonti A, Chen Z, Gao Z *et al*. (2007). NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN. *Cell* **128**: 129–139.

Zhu F, Zykova TA, Kang BS, Wang Z, Ebeling MC, Abe Y *et al*. (2007). Bidirectional signals transduced by TOPK-ERK interaction increase tumorigenesis of HCT116 colorectal cancer cells. *Gastroenterology* **133**: 219–231.

Zlobec I, Molinari F, Kovac M, Bihl MP, Altermatt HJ, Diebold J *et al*. (2010). Prognostic and predictive value of TOPK stratified by KRAS and BRAF gene alterations in sporadic, hereditary and metastatic colorectal cancer patients. *Br J Cancer* **102**: 151–161.

Zykova TA, Zhu F, Lu C, Higgins L, Tatsumi Y, Abe Y *et al*. (2006). Lymphokine-activated killer T-cell-originated protein kinase phosphorylation of histone H2AX prevents arsenite-induced apoptosis in RPMI7951 melanoma cells. *Clin Cancer Res* **12**: 6884–6893.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)