PRKACB is downregulated in non-small cell lung cancer and exogenous PRKACB inhibits proliferation and invasion of LTEP-A2 cells

YONG CHEN\textsuperscript{1}, YING GAO\textsuperscript{2}, YE TIAN\textsuperscript{1} and DA-LI TIAN\textsuperscript{1}

Departments of\textsuperscript{1} Thoracic Surgery and\textsuperscript{2} Pathology, The Fourth Affiliated Hospital of China Medical University, Shenyang, Liaoning 110032, P.R. China

Received November 21, 2012; Accepted March 20, 2013

DOI: 10.3892/ol.2013.1294


corresponding normal tissues of 30 cases, using quantitative RT-PCR and western blot analysis. A plasmid containing full-length PRKACB was transfected into LTEP-A2 cells to further investigate the effects of PRKACB overexpression on proliferation, apoptosis and invasion of the transfected cells, which were examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), colony formation, flow cytometry and Transwell assays. The results revealed that the NSCLC tissues exhibited much lower levels of PRKACB mRNA and protein compared with their corresponding normal tissues. The upregulation of PRKACB decreased the numbers of proliferative, colony and invasive cells, while the apoptotic rates of transfected cells were increased. These data indicate that PRKACB is downregulated in NSCLC tissues and that upregulation of PRKACB may be an effective way to prevent the progression of NSCLC.

Introduction

Lung cancer is the most commonly diagnosed type of cancer in males and the leading cause of cancer mortality in each gender in economically developed and developing countries (1). Non-small cell lung carcinoma (NSCLC) accounted for ~85% of the all lung cancer cases (2). Standard lung cancer treatment modalities include surgery, chemotherapy, targeted therapy and radiation therapy; however, not all patients benefit from routine therapy. The overall 5-year survival rate of lung cancer patients remains relatively low at ~15% (2). Therefore, the identification of useful biomarkers and exploration of novel therapeutic targets are necessary and demanding tasks.

The protein kinase cAMP-dependent catalytic \(\beta\) (PRKACB) gene is located at chromosome site 1p31.1 and encodes cAMP-dependent protein kinase A (PKA) catalytic subunit \(\beta\). The PRKACB protein is a member of the Ser/Thr protein kinase family and a key effector of the cAMP/PKA-induced signal pathway that is involved in numerous cellular processes, including cell proliferation, apoptosis, gene transcription, metabolism and differentiation (3). Typically, PKA is an inactive holoenzyme consisting of two catalytic (C) subunits bound to a regulatory (R) subunit dimer. When four cAMP molecules bind the R subunits, the C subunits are released (4) and free active catalytic subunits phosphorylate serine and threonine residues on specific substrate proteins, which include C-Raf, RhoA, Src and CUTFI, that are involved in cellular proliferation, apoptosis, gene transcription, metabolism and differentiation (5-8). In the human enzyme, four different R subunits (RI\(\alpha\), RI\(\beta\), RII\(\alpha\) and RII\(\beta\)) and four different C subunits (C\(\alpha\), C\(\beta\), C\(\gamma\) and PrKX) have been identified (3). In total, ten different splice variants encoded by the PRKACB gene have been found and a certain number of these were revealed to be expressed in human brain, lymphoid and neuronal tissues (9-11). Multiple PRKACB subunits have also been observed in human prostate specimens and it appears that the PRKACB variants play varying roles in proliferation and differentiation of prostate cancer progression (12). It has been demonstrated that transcription of PRKACB may be directly activated by c-MYC, which is associated with tumorigenesis by the promotion of cell proliferation (13). It has also been

Correspondence to: Professor Da-Li Tian, Department of Thoracic Surgery, The Fourth Affiliated Hospital of China Medical University, No. 4 ChongShan East Road, Shenyang, Liaoning 110032, P.R. China

E-mail: dalitian@hotmail.com

Key words: protein kinase cAMP-dependent catalytic \(\beta\), non-small cell lung cancer, proliferation, apoptosis, invasion
shown that a variant of PRKACB phosphorylates the p75
neuropilin receptor (p75NTR) and regulates its localization
to lipid rafts (14). PRKACB was identified as a candidate gene
that is directly or indirectly involved in apoptosis in human
mantle cell lymphoma (MCL) tumors (15). In addition, a novel
interaction between PRKACB, the cell cycle and apoptosis
regulatory protein-1 (CARP-1) was identified and confirmed
by glutathione-S-transferase (GST) pull-down experiments in
brain tissue (16). However, limited information is known with
regard to its expression and role in human NSCLC.

The present study aimed to assess the role of PRKACB
in the development and progression of human NSCLC. The
mRNA and protein expression patterns of PRKACB were first
examined in the NSCLC and corresponding normal tissues.
Moreover, plasmid vectors containing full-length PRKACB
and transfected human adenocarcinoma LTEP-A2 cells were
constructed to increase the PRKACB expression. The effects
of PRKACB upregulation on cell proliferation, clonogenicity,
apoptosis and invasion were then investigated in the LTEP-A2
cells.

Materials and methods

Tissue samples and patients. NSCLC tissues (12 cases of lung
squamous cell carcinoma tissues, 18 cases of lung adenocarci-
noma tissues; 22 of these 30 cases presented with lymph node
metastasis) and their corresponding normal tissues (30 cases)
were collected from 30 patients who underwent surgery at
the Department of Thoracic Surgery, The Fourth Affiliated
Hospital of China Medical University, Shenyang, Liaoning,
China, between 2008 and 2012. All tumor tissues were diag-
nosed histopathologically by at least two trained pathologists.
Written informed consent was obtained from all patients
prior to surgery and the study protocol was approved by the
Institutional Review Board for the use of Human Subjects
at China Medical University (Shenyang, China). None of the
patients received pre-operative chemotherapy or radiation
therapy. Surgically-removed tumors and matched normal
tissues were immediately frozen in liquid nitrogen and kept at
-80°C until the extraction of the RNA and protein.

DNA extraction and real-time RT-PCR. Total RNA from the
frozen tissues was isolated using TRIzol reagent (Takara Bio
Inc., Dalian, Liaoning, China). Quantitative real-time poly-
merase chain reaction (QPCR) was conducted using SYBR
Premix Ex Taq (Takara Bio Inc.) in a total volume of 20 µl
using a 7300 Real-Time PCR System (Applied Biosystems,
Foster City, CA, USA), according to the manufacturer's instruc-
tions. The DNR Imaging System (DNR Bio-Imaging Systems, Israel) was used to iden-
tify the specific bands, and the optical density of each band
was measured using Image J software (NIH, Bethesda, MD,
USA). The ratio between the integrated optical density (IOD) of
PRKACB and GAPDH of the same sample was calculated as the
relative content and expressed graphically.

Cell culture and transfection. Lung adenocarcinoma LTEP-A2
cells were obtained from the Shanghai Cell Bank (Shanghai,
China). The cells were grown in RPMI-1640, supplemented
with 10% fetal bovine serum (FBS; Hyclone, USA) and placed
in an incubator with 5% CO2 at 37°C. To increase the PRKACB
expression for subsequent experiments, the LTEP-A2 cells
were transfected with a plasmid containing full-length PRKACB
(pEGFP-C1-PRKACB) and the vector control (pEGFP-C1; Takara Bio Inc.) for 48 h using
Lipofectamine LTX with PLUS reagent (Invitrogen, Carlsbad,
CA, USA), according to the manufacturer's instructions. The
experiments were repeated at least three times. The efficiency
of the transfection in the experiments was ≥50%. Following
36-48 h of transfection, the cells with high PRKACB expres-
sion were confirmed by real-time RT-PCR and western blot
analysis.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
(MTT) assay. The MTT assay was used to evaluate the prolif-
eration of the transfected cells. The cells were detached and
seeded into 96-well plates (5x103 cells/100µl/well) in
parallel and transfected with PRKACB and the vector control.
During the following 4 days, the absorbance of one indicated
plate was examined each day, and the cells in the other plates
were cultured continuously. A total of 20 µl MTT (5 mg/ml)
was added to each well of the indicated plate, and 4 h later the
liquids were removed and 150 µl dimethyl sulfoxide (DMSO)
was added. Following 10 min of agitation, the absorbance was
measured using a microplate reader (TECAN, Männedorf,
Switzerland) at 492 nm. The results were plotted as the
mean ± SD of five determinations.

Colonies formation assay. The cells were transfected with
PRKACB and the vector for 24 h. Thereafter, 200 cells
were planted into 6-cm cell culture dishes and incubated for
14 days. The plates were stained with Giemsa, and colonies
with >50 cells were counted.

Western blot analysis. The total protein from the frozen tissues
was extracted in a lysis buffer (Beyotime Biotechnology, Haimen,
Jiangsu, China) and the protein content was determined using
the bicinchoninic acid (BCA) assay (Beyotime Biotechnology).
A total of 80 µg total protein was separated by sodium dodecyl
sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then
transferred onto polyvinylidene fluoride (PVDF) membranes.
Subsequent to blocking with 5% bovine serum albumin (BSA),
PRKACB antibody (1:500; Santa Cruz) and GAPDH antibody
(1:500; Santa Cruz) were incubated on membranes for PRKACB
and GAPDH protein overnight at 4°C. The membranes were
then incubated for 2 h at 37°C with goat anti-rabbit IgG (1:4000;
Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China).
Immunoreactive strips were identified using the enhanced
chemiluminescence (ECL) system (Beyotime Biotechnology)
following the manufacturer's instructions. The DNR Imaging
System (DNR Bio-Imaging Systems, Israel) was used to iden-
tify the specific bands, and the optical density of each band
was measured using Image J software (NIH, Bethesda, MD,
USA). The ratio between the integrated optical density (IOD) of
PRKACB and GAPDH of the same sample was calculated as the
relative content and expressed graphically.

Cell cycle and apoptosis. Cell cycle and apoptosis in the LTEP-A2
and transfected human adenocarcinoma LTEP-A2 cells were
constructed to increase the PRKACB expression. The effects
of PRKACB upregulation on cell proliferation, clonogenicity,
apoptosis and invasion were then investigated in the LTEP-A2
cells.
Cell apoptosis assay. Cell apoptosis was examined by flow cytometry using an Annexin V-PE/7-aminoactinomycin D (7-AAD) apoptosis detection kit (KeyGEN Biotech., Nanjing, China), following the manufacturer's instructions. At 24 h post-transfection, the cells were washed twice in ice-cold PBS. The cells (100 µl; 1x10^5) were gently mixed with 50 µl binding buffer and 5 µl 7-AAD and then incubated for 15 min at room temperature in the dark. Subsequent to supplementation with another 450 µl binding buffer, 1 µl Annexin V-PE was added to the cells, which were then incubated for another 15 min at room temperature in the dark. Cell apoptosis was detected using a flow cytometer. The results are representative of three individual experiments.

Cell invasion assay. The cell invasion assay was performed using a 24-well Transwell chamber (Costar, Cambridge, MA, USA). At 24 h post-transfection, the cells (4x10^4) were seeded in the upper chamber of a 8-µm pore size insert pre-coated with Matrigel (BD Biosciences-Pharmingen, San Diego, CA, USA), and cultured in RPMI-1640 without FBS for a further 24 h. The cells were allowed to migrate towards the medium containing 10% FBS in the bottom chamber. The non-migratory cells on the upper membrane surface were removed with a cotton tip, and the migratory cells attached to the lower membrane surface were fixed with 4% paraformaldehyde and stained with crystal violet (Sigma, St. Louis, MO, USA). The number of invaded cells were counted in 10 randomly selected power fields under a microscope (magnification, x200) (Olympus CK30; Olympus, Tokyo, Japan). The experiments were performed in triplicate.

Statistical analysis. The SPSS for Windows version 17.0 statistical analysis software (SPSS, Inc., Chicago, IL, USA) was applied to complete the data processing. A paired-samples t-test was used to compare the differences between the PRKACB expression in the NSCLC and corresponding normal tissues. One-way ANOVA was used to compare the differences in PRKACB expression in the transfected LTEP-A2 cells or controls. All data are represented as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of PRKACB mRNA and protein in human NSCLC tissues and their corresponding normal tissues. The PRKACB mRNA expression was first quantitatively determined in the clinical samples using real-time RT-PCR. Of the 30 patients, 25 (83.3%) demonstrated a lower expression level of PRKACB mRNA in the NSCLC tissues compared with the corresponding normal tissues (Fig. 1A). In addition, the mean expression value of the PRKACB mRNA in NSCLC tissues (relative ratio of PRKACB/GAPDH; 0.007677±0.004608) was significantly weaker than the value in the normal tissues (0.031936±0.018996; P<0.05; Fig. 1B). Consistent with the mRNA level, the protein levels of PRKACB were downregulated in the NSCLC tissues compared with the normal tissues (0.350±0.124 vs. 0.964±0.245, respectively; P<0.05; Fig. 1C). The study also demonstrated that PRKACB protein expression was downregulated in lymph node metastasis tissues (data not shown).

PRKACB upregulation inhibits proliferation and clonogenicity in NSCLC cells. To elucidate the biological role of PRKACB during carcinogenesis, the physiological effects of PRKACB upregulation on cell proliferation and clonogenicity were examined using the LTEP-A2 cells. Fig. 2A shows the overexpression of PRKACB in the transfected cells. The study showed that 3 days after PRKACB transfection, the absorbance values in the PRKACB, vector and control groups were 0.93±0.08, 1.41±0.12 and 1.36±0.09, respectively (one-way ANOVA, P<0.05). The growth curve shows that the cells transfected with pEGFP-C1-PRKACB grew more slowly than the empty vector-transfected cells and control group cells, indicating that PRKACB inhibits proliferation in NSCLC cells (Fig. 2B). The colony formation efficiencies of the LTEP-A2 cells transfected with PRKACB and the vector control for 24 h were...
compared next. In total, 200 cells were planted on 6-cm cell culture dishes. At two weeks post-transfection, the plates were stained with Giemsa and colonies with >50 cells were counted. The numbers of cell colonies in the PRKACB, vector and control groups were 23.42±5.38, 89.28±7.15 and 86.85±6.86, respectively (one-way ANOVA, P<0.05; Fig. 2C). These results showed that the increased expression of PRKACB significantly inhibited the colony formation efficiencies of the LTEP-A2 cells. Collectively, these data suggest that PRKACB may act as a negative regulator of cell growth and that its downregulation plays a significant role in NSCLC carcinogenesis.

*Elevated apoptotic rate in PRKACB transfected cells.* PRKACB has been considered to prevent the overgrowth of cells by inducing cell apoptosis (15,16). Therefore, apoptosis was examined following PRKACB transfection using Annexin V-PE/7-AAD assay and flow cytometry. It was confirmed that PRKACB was upregulated in the transfected cells.
The apoptotic rates of the LTEP-A2 cells in the PRKACB, vector and control groups were 24.43±3.42, 4.39±1.63 and 3.48±1.44%, respectively (one-way ANOVA, P<0.05; Fig. 3). The results showed that apoptosis was significantly induced in the PRKACB overexpressed cells.

Effect of PRKACB upregulation on the invasive potential of transfected cells. It has been acknowledged that PKA may inhibit RhoA signaling, which has been implicated in the process of tumor cell invasion and metastasis (6). To determine whether PRKACB expression further affects the invasion of LTEP-A2, the present study compared the invasive ability of the three cell groups. The number of invasive cells in the PRKACB, vector and control groups were 83.6±9.5, 156.9±13.7 and 154.2±12.9, respectively (one-way ANOVA, P<0.05; Fig. 4). These results show that the increased expression of PRKACB significantly inhibited the invasion of the LTEP-A2 cells, as demonstrated by the Matrigel invasion assay.

Discussion

The PRKACB gene is located at the 1p31.1 chromosome site and encodes PKA catalytic subunit β, which is a member of the Ser/Thr protein kinase family. As a key effector of the cAMP/PKA-induced signaling pathway, the free C subunits phosphorylate serine and threonine residues on specific substrate proteins and regulate a wide range of cellular processes. Previous studies have identified the loss of 1p31.1 in MCL patients and the MCL cell line. PRKACB has been identified as an apoptotic candidate gene and it appears that decreased expression of PRKACB is implicated in human MCL (15). PRKACB tissue-specific expression has also been found in human brain, neuronal, lymphoid and prostate cancer tissues, and has been reported to be correlated with cellular proliferation or differentiation processes (9-12). However, there are no studies investigating the role of PRKACB in lung cancer. In the present study, the mRNA and protein levels of PRKACB were downregulated in the human NSCLC tissues compared with their corresponding normal tissues. These results suggest that PRKACB has a critical effect in the tumorigenesis and aggression of NSCLC.

A recent study discovered a novel interaction between PRKACB, the cell cycle and CARP-1; this was confirmed by GST pull-down experiments in brain tissue (16). A study has also demonstrated that PRKACB interacts with p75NTR, which phosphorylates p75NTR at Ser304 (14). In the majority of cases, the most prominent biological function of p75NTR is that it induces cell death and induces the activity of the JNK-p53-Bax apoptosis pathway and other proteins that regulate cell death, such as NRIF (17). PKA-mediated phosphorylation at Ser304 has been shown to promote the translocation of p75NTR to lipid rafts and to regulate the downstream signals of p75NTR, including the inactivation of RhoA, which has been implicated in the process of tumor cell invasion and metastasis. In addition, PKA may also directly inhibit RhoA signaling; when Ser188 is phosphorylated, RhoA becomes inactive and thereby induces characteristic morphological changes, causing cell rounding (6). These data suggest that decreased PRKACB is associated with cellular apoptosis, invasion and metastasis.

With the aim of assessing the role of PRKACB in the development and progress of human NSCLC, the present study examined the effects of exogenously-transfected PRKACB on the apoptosis and invasion of LTEP-A2 cells. Consistent with the aforementioned findings, the present study concluded that the upregulation of PRKACB increased the number of apoptotic cells and decreased the number of invasive cells. The results demonstrate the potential role of PRKACB in the development and progression of human NSCLC.

As previously described, PKA was able to induce the signal pathway that is involved in numerous cellular process, including cell proliferation, apoptosis and gene transcription (3). cAMP-mediated PKA activation has been shown to have anti-proliferative effects in a number of cell types, including thyroid papillary carcinoma, ovarian epithelial cancer, breast cancer and malignant glioma cells (18-26). These anti-proliferative effects are mainly associated with the negative regulation of the Ras-Raf-MEK-ERK signaling pathway by interfering with the activation of Raf-1 directly or via Ras in the Raf-1 pathway (5,24,27). Several other mechanisms have been proposed to explain the anti-proliferative effects of activated PKA on various other cells and tissues, including a decrease in the expression level of cyclin D3 and an upregulation of the amount of p27kip1 (26). PKA is able to inhibit CUTL1-mediated proliferation and migration (8), as well as the LPA stimulation of SRF by promoting the dissolution of F-actin (19). In this study, we further examined the effects of exogenously transfected PRKACB on the proliferation of LTEP-A2 cells. The observation that the upregulation of PRKACB induces decreased proliferation of the LTEP-A2 cells is consistent with a negative role for PKA in the proliferation of these cells. Exogenously expressed PRKACB may effectively inhibit the progression of lung cancer. However, the fact that the excess of free PRKACB subunits may generate signals different from those generated by the cAMP/PKA-induced signal pathway cannot be excluded. It has also been previously shown that the activation of PKA has either proliferative or anti-apoptotic effects in cultured cells, and that these opposite responses may be due to the existence of cell type-specific targets of this signaling pathway (12,13).

The present study demonstrated that PRKACB was downregulated in human NSCLC tissues. Decreased PRKACB appears to be associated with cellular apoptosis, invasion and proliferation. However, the molecular mechanisms for these processes remain primarily unknown. Increased PRKACB expression is possibly an effective inhibitor of lung cancer. The upregulation of PRKACB may provide a useful strategy for future NSCLC inhibitory therapies.

Acknowledgements

This study was supported by the National Nature Science Foundation of China (30973502).

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
2. Molina JR, Yang P, Cassivi SD, Schild SE and Adjei AA: Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 83: 584-594, 2008.
15. Korbin JD, Sugden PH, West L, Flockhart DA, Lincoln TM and McCarthy D: Studies on the properties and mode of action of the purified regulatory subunit of bovine heart adenosine 3'-5'-monophosphate-dependent protein kinase. J Biol Chem 253: 3997-4003, 1978.

16. Schraders M, Jares P, Bea S, Schoenmakers EF, et al: Integrated genomic and expression profiling in mantle cell lymphoma: identification of gene-dosage regulated candidate genes. Br J Haematol 143: 210-221, 2008.

17. Kaplan DR and Miller FD: Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10: 381-391, 2000.

18. Matsumoto H, Sukamoto A, Fujimura M, et al: Cyclic AMP-mediated growth suppression and MAPK phosphorylation in thyroid papillary carcinoma cells. Mol Med Rep 1: 245-249, 2008.

19. Nguyen GH, French R and Radhakrishna H: Protein kinase A inhibits lysophosphatidic acid induction of serum response factor via alterations in the actin cytoskeleton. Cell Signal 16: 1141-1151, 2004.

20. Chen TC, Hinton DR, Zidovetzki R and Hofman FM: Up-regulation of the cAMP/PKA pathway inhibits proliferation, induces differentiation, and leads to apoptosis in malignant gliomas, Lab Invest 78: 165-174, 1998.

21. Cassoni P, Sapino A, Fortunati N, Munaron L, Chini B and Busolati G: Oxytocin inhibits the proliferation of MDA-MB231 human breast-cancer cells via cyclic adenosine monophosphate and protein kinase A. Int J Cancer 72: 340-344, 1997.

22. Hewer RC, Sala-Newby GB, Wu YJ, Newby AC and Bond M: PKA and Epac synergistically inhibit smooth muscle cell proliferation. J Mol Cell Cardiol 50: 87-98, 2011.

23. Liu J, Li XD, Ora A, Heikkilä P, Vaheri A and Vuolteinen R: cAMP-dependent protein kinase activation inhibits proliferation and enhances apoptotic effect of tumor necrosis factor-alpha in NCI-H295R adrenocortical cells. J Mol Endocrinol 33: 511-522, 2004.

24. D'Angelo G, Lee H and Weiner RI: cAMP-dependent protein kinase inhibits the mitogenic action of vascular endothelial growth factor and fibroblast growth factor in capillary endothelial cells by blocking Raf activation. J Cell Biochem 67: 353-366, 1997.

25. Hordijk PL, Verlaan I, Jalink K, van Corven EJ and Moollenar WH: cAMP abrogates the p21ras-mitogen-activated protein kinase pathway in fibroblasts. J Biol Chem 269: 3534-3538, 1994.

26. van Oirschot BA, Stahl M, Lens SM and Medema RH: Protein kinase A regulates expression of p27(kip1) and cyclin D3 to suppress proliferation of leukemic T cell lines. J Biol Chem 276: 33854-33860, 2001.

27. Al-Wadei HA and Schuller HM: Cyclic adenosine monophosphate-dependent cell type-specific modulation of mitogenic signaling by retinoids in normal and neoplastic lung cells. Cancer Detect Prev 30: 403-411, 2006.