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Invasiveness and anchorage independent growth ability augmented by PTEN inactivation through the PI3K/AKT/NFkB pathway in lung cancer cells

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ABSTRACT

PTEN is inactivated in a subset of lung cancer; therefore, we investigated the involvement of PTEN inactivation in invasiveness of lung cancer cells. AKT at Ser473 was phosphorylated in several lung cancer cell lines with loss of PTEN expression. Therefore, we created a tetracycline inducible expression system of wild-type PTEN (PTEN-WT) as well as catalytically (PTEN-G129R) and lipid phosphatase (PTEN-G129E) inactive PTEN mutants using the PC14, PC9 and PC3 lung adenocarcinoma cell lines, in which endogenous PTEN expression was not detected and AKT at Ser473 was phosphorylated by Western blot analysis. Induction of PTEN-WT reduced phosphorylation of AKT and inhibited the transcriptional activity of NFkB, whereas PTEN mutants did not, suggesting that PTEN inactivation results in the activation of the AKT/NFkB pathway in PC14, PC9 and PC3 cells. Furthermore, overexpression of PTEN-WT suppressed anchorage independent growth in soft agar and reduced invasiveness in a trans-well chamber assay of PC14 cells. Neither PTEN-G129R nor PTEN-G129E had suppressive effects on anchorage independent growth and invasiveness. Augmentation of invasiveness by constitutively active AKT was also shown in mouse NIH3T3 cells. Therefore, it was strongly indicated that activation of the PI3K/AKT/NFkB pathway by PTEN inactivation results in augmented invasiveness in lung cancer cells and lipid phosphatase activity of PTEN plays a key role in this process.

1. Introduction

The PTEN (phosphates and tensin homologue deleted on chromosome 10) tumor suppressor gene is frequently deleted or mutated in a wide variety of human cancers, including glioblastoma [1], melanoma [2], prostate cancer [3], breast cancer [4], lung cancer [5], and endometrial cancer [6]. In addition, PTEN germline mutations are responsible for the development of Cowden disease, Bannayan-Zonana syndrome [7,8] and Lhermitte Duclos disease, in which disorganized hamartomas appear in various organs [9]. Besides functioning as a tumor suppressor, PTEN is also essential for embryonic development [10,11]. PTEN encodes a protein that has sequence homology with phosphatases which dephosphorylate both tyrosine and serine/threonine phosphates on proteins [12–14]. PTEN is also capable of dephosphorylating inositol phospholipids [15]. In particular, PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-4.5-biphosphate (PIP2). Formation of these lipids is catalyzed by phosphoinositol 3-kinase (PI3K) upon stimulation with growth factors [16]. PIP2 and PIP3 are both involved in the PI3K signaling pathway [15,17–19]. One important downstream target of PI3K is the AKT serine/threonine kinase [19]. AKT is recruited to the plasma membrane by PIP3, and then phosphorylated at Thr308 by protein dependent kinase 1 (PDK1). For full activation, AKT needs to be subsequently phosphorylated at Ser473 by PDK2. Activated AKT leaves the membrane and phosphorylates its downstream targets such as Bad [20], IKKalpha and mTOR [20,21]. AKT activation induces expression of the anti-apoptotic Bcl-2, activation of NFkB, inhibition of Forkhead family of transcription factors, and inactivation of pro-apoptotic Bad [22–25]. AKT-mediated modifications of above target genes ultimately result in cellular transformation, survival, proliferation and invasion of a variety of cancers [26–29].

Lung cancer is the leading cause of cancer death in the world [30], and approximately 90% of patients with lung cancer ultimately die from metastatic disease [31]. Metastasis is the end result of a complex series of steps involving multiple tumor-host interactions [32]. One important step in this process is invasion of cancer cells. PTEN is inactivated in a subset of and Akt is activated frequently in lung cancer. However, it is unknown whether PTEN plays any role in lung cancer cell invasion and whether such an effect is mediated through the PI3K/AKT/NFkB pathway. To address this question, we first screened several lung cancer cell lines for the status of PTEN and AKT. We then constructed a PTEN inducible system using the

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PC14 lung cancer cell line, in which endogenous PTEN is absent and AKT is phosphorylated. The results indicate that PTEN inactivation plays an important role in the invasiveness of lung cancer cells and this process is mediated by the PI3K/AKT/NF-kB pathway.

2. Materials and methods

2.1. Cell culture

PC3, PC9 and PC14 cells were cultured in RPMI-1640 medium (Sigma Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37 °C in a humidified incubator with 5% CO_2.

2.2. Construction of PTEN expression vectors and transfection

PTEN cDNA was cloned as described by Myers et al. [33]. Briefly, total RNA was extracted from A549 cells using Trizol (Life Technologies), and the first-strand cDNA was synthesized using oligo(dT) and Superscript RNaseH2 RT, according to the manufacturer's protocol (Life Technologies). PTEN cDNA was amplified using forward (5′-CGCGAATTCGCCATGGCAGCCATCATCAAAGAGATCGTTAGCAG-AAACAAAAGGAGATATCAAGAGGATGGATTCGACTTAGAC-3′) and reverse (5′-CGCGAATTCTCAGACTTTTGTAATTTGTGTATGCTGATCT-TCATCAAAAGGTTCATTCTCTGGATCAGAGTCAGTGGAGGTGTCAGA-3′) primers and Pfu DNA polymerase (Stratagene), and amplified cDNA was cloned into pcDNA 3 and a tetracycline inducible vector pcDNA4/TO. The PTEN-G129R and PTEN-G129E mutants were generated by site-directed mutagenesis. By using FUGEN (Roche), PC14 cells were first stably transfected with a regulatory plasmid pcDNA6/TK, and clones resistant to blasticidin (10 μg/ml) were then stably transfected with the pcDNA4/TO-PTEN-WT, pcDNA4/TO-PTEN-G129R, and pcDNA4/TO-PTEN-G129E expression vectors. Double transfectants were selected in the medium containing blasticidin (10 μg/ml) and zeocin (50 μg/ml). PTEN expression was induced by addition of tetracycline (2 μg/ml) and was verified by Western blot analysis. pcDNA-PTEN-WT, pcDNA-PTEN-G129R and pcDNA-PTEN-G129E vectors used for transient transfection studies.

2.3. Western blot analysis

Cell lysates were prepared in ice-cold RIPA buffer (10 mM Tris–HCl pH7.5, 150 mM NaCl, 2 mM EDTA, 1% NP40, 1% sodium deoxycholate, 0.1% SDS). Cellular debris was removed by centrifugation at 12,000 × *g* for 5 min at 4 °C. One hundred μg of proteins were subjected to SDS–PAGE using 2–15% or 7.5% polyacrylamide gels (PIERCE), proteins were immunoblotted onto Hybond-PVDF membrane (Amersham-PharmaciaBiotech), and labeled with antibodies. AKT and phospho-AKT (Ser473) antibodies were obtained from Cell Signaling Technology. PTEN, GAPDH, Tubulin and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Primary and secondary antibody labeling blots were treated with Super Signal West Pico chemiluminescent substrate (Pierce), exposed to Hyperfilm ECL (Amersham-PharmaciaBiotech), and developed.

2.4. Proliferation assay

Mock, pcDNA4/TO-PTEN-WT, pcDNA4/TO-PTEN-G129R, and pcDNA4/TO-PTEN-G129E transfected PC14 cells were seeded into 96-well plates (7.5×10^4 cells/ml) and treated with tetracyclin (2 μg/ml) for 24 h. At the end of the incubation period, cell numbers were counted by a luminometric method using a CytotoxGlo kit (Promega).

2.5. Luciferase reporter assay

Mock, pcDNA4/TO-PTEN-WT, pcDNA4/TO-PTEN-G129R, pcDNA4/TO-PTEN-G129E stably transfected PC14 cells were transfected with pGL3-NFkB-promoter-Luc (0.5 μg/well) and with pRL-*Renilla* (0.05 μg/well). For PC9 and PC3 cells; Mock, pcDNA4/TO-PTEN-WT, pcDNA4/TO-PTEN-G129R, pcDNA4/TO-PTEN-G129E transiently co-transfected with pGL3-NFkB-promoter-Luc (0.5 μg/well) and with pRL-*Renilla* (0.05 μg/well). Following transfection, cells were treated with tetracyclin (2 μg/ml) and cultured for an additional 24 h. Luciferase activities were then measured using a luciferase Assay System Kit (Promega).

2.6. Soft agar colony assay

Anchorage independent growth was determined by a soft agar colony assay as described [34]. Briefly, 5×10^4 cells were prepared in 3 ml of medium containing 0.4% noble agar (Difco), and layered onto 5 ml of bottom layer containing 0.5% noble agar (Difco). Two μg/ml tetracycline was added to both layers. Plates were incubated for 2–3 weeks, developed colonies were fixed and stained with HBSS containing 1.5% glutaraldehyde and 0.06% methylene blue, and visible colonies were counted.

2.7. Cell invasion assay

Cell invasion assays were carried out using a trans-well matrigel invasion chamber (Becton Dickinson Labware). The chambers were hydrated with 1% BSA in RPMI-1640 for 1 h at room temperature, then placed into 24-well tissue culture plates containing 750 μl of RPMI-1640 with 10% FCS. For invasion study, we transiently transfected PC9 and PC3 cells with pcDNA-PTEN-WT, pcDNA-PTEN-G129R, pcDNA-PTEN-G129E and stably trasnfected PC14 cells with pcDNA4/TO-PTEN-WT, pcDNA4/TO-PTEN-G129R, pcDNA4/TO-PTEN-G129E expression vectors. All cells were trypsinized, washed twice in PBS, and 1×10^4 cells were added to each trans-well chamber and allowed to migrate to the bottom chamber for 24 h. Two μg/ml tetracycline was added to both chambers, After 24 h, cells on the upper surface of the filter were then removed by wiping with a cotton swab, and the number of migrated cells was determined. The extent of migration was expressed by normalizing the number of PTEN-WT, PTEN-G129R and PTEN-G129E expressing cells by that of empty vector transfected cells. Ten individual wells were counted for every experiment. Error bars representing S.E. and *P*s were calculated by repeated measurements of ANOVA (Graphpad Software), followed by a Student–Newman–Keuls test.

3. Results

3.1. Effects of PTEN expression on the PI3K/AKT/NFkB pathway in lung cancer cells

We first examined expression levels of PTEN in 9 lung cancer cell lines and in an immortalized small airway epithelial cell line, hSAEC-T1 [35], by Western blot analysis (Fig. 1). PTEN expression was detected in H23, II-18, KTSQ-1 and PC13 cells, but the amounts of PTEN protein in these cell lines were slightly lower than that in hSAEC-T1 cells. PTEN was not detected in the remaining five cell lines, PC14, PC9, PC3, PC10 and H1299, suggesting that PTEN is inactivated in these cell lines. It was previously reported that both allele of PTEN gene is deleted in PC10 and its promoter is methylated in PC14 and H1299 cells [5,36,37]. Consistent with these findings we did not observe any PTEN expression in these cells. Since AKT is downstream of the PTEN pathway and is often phosphorylated in

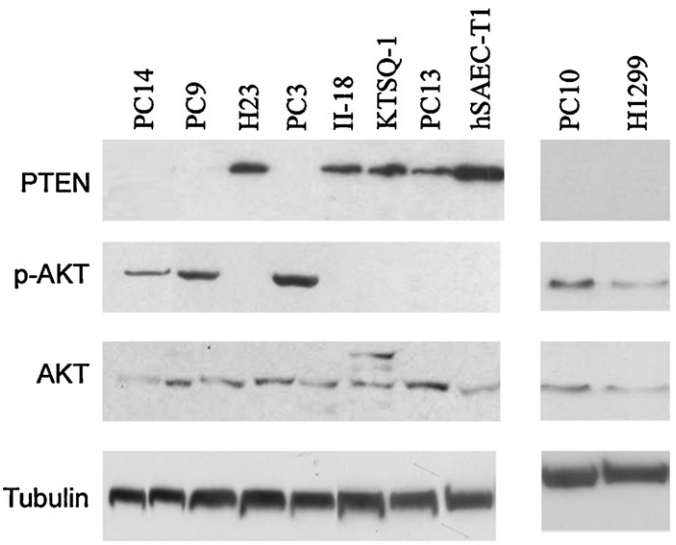

Fig. 1. Determining the levels of PTEN, phospho-AKT, and AKT in 9 lung cancer cell lines and an immortalized small airway epithelial cell line. Western blot analysis of PTEN, p-AKT and AKT protein expression in 9 NSCLC cell lines and one immortal lung epithelial cell. All cells were cultured in included in RPMI-1640 containing 10% FCS. Cell were lysed and then 100 microgram protein were fractionated on 4–20% gradient gel. Proteins were blotted onto PVDF membrane and labeled against indicated antibodies.

cells with PTEN inactivation, we next investigated the expression and phosphorylation of AKT in these cell lines. AKT was detected in all the cell lines examined. AKT at Ser473 was phosphorylated in five cell lines, PC14, PC9, PC3, PC10 and H1299, all of which showed the absence of PTEN protein. Therefore, it was strongly indicated that PTEN inactivation results in the activation of AKT in these lung cancer cell lines.

To examine the effect of PTEN expression in lung cancer cells, tetracycline-inducible PTEN vectors were constructed and transfected into PC14 cells, in which endogenous PTEN expression was not detected by Western blot analysis. Vectors carrying wild-type PTEN (PTEN-WT) as well as catalytically (PTEN-G129R) and lipid phosphatase (PTEN-G129E) inactive PTEN mutants were prepared for this study as described in Materials and Methods. These vectors were stably transfected into PC14 cells, transiently transfected in PC9 and PC3 cells, and PTEN induction after tetracycline treatment was confirmed by Western blot analysis. Representative results of PTEN induction are shown in Fig. 2A. Wild-type PTEN expression was detected 4 h after tetracycline treatment, reached the maximum level at 24 h, and continued up to 120 h after the treatment. We were also able to obtain PTEN-G129R and PTEN-G129E mutant-inducible cells. Induction of PTEN after 24 and 48 h of tetracycline treatment in PTEN-WT, PTEN-G129R and PTEN-G129E transfected cells are shown in Fig. 2B.

We next examined the effects of induced PTEN expression on phosphorylation levels of AKT in PC14 cells (Fig. 2B). The amount of phosphorylated AKT was decreased at both 24 h and 48 h after tetracycline treatment in PTEN-WT transfectants. In contrast, such a decrease was not observed in mock, PTEN-G129R and PTEN-G129E transfectants. Therefore, AKT phosphorylation was confirmed to be partly due to PTEN inactivation in PC14 cells.

NFkB is a downstream target of the PI3K/AKT pathway; therefore, we next examined the effects of PTEN expression on NFkB activity by a reporter assay. Cells expressing PTEN-WT, PTEN-G129R and PTEN-G129E were transiently transfected with an NFkB luciferase reporter vector and a renilla luciferase vector as internal control. Expression of wild type PTEN significantly inhibited NFkB activity; however, expression of PTEN mutants did not (Fig. 2C). These results indicate that PTEN can suppress the AKT/NFkB pathway through its lipid phosphatase activity in PC14 cells.

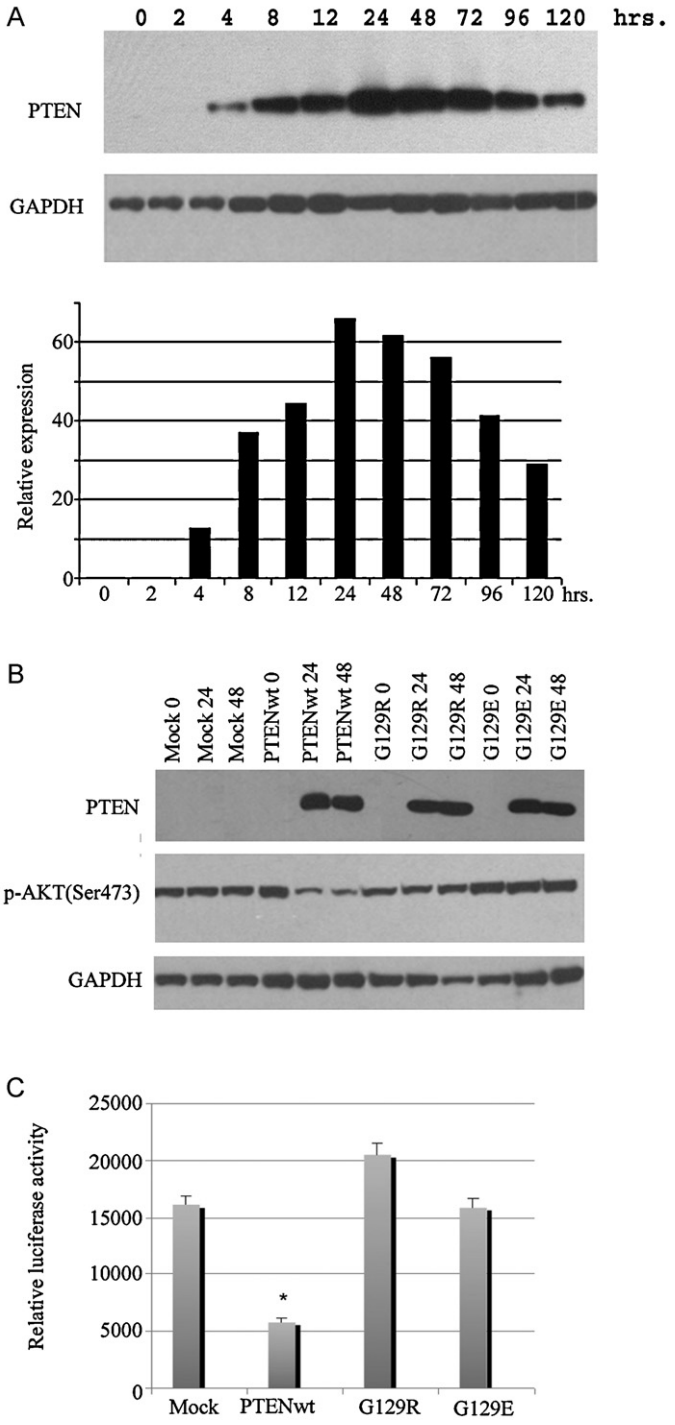

Fig. 2. Ectopic expression of PTEN and its effect on AKT phosphorylation and NFkB activity on PC 14 cells. Cells stably transfected with PTEN-WT expression vector were treated with tetracycline (2 μg/ml) (A). Expressions of PTEN, p-AKT, and AKT were determined 0, 24 and 48 h after tetracycline treatment in mock, PTEN-WT, PTEN-G129R and PTEN-G129E transfected PC 14 cells (B). The effects of PTEN-WT, PTEN-G129R and PTEN-G129E expression on NFkB activity (C). Error bars indicate S.D., $^{*}p < 0.05$ comparing PTENwt transfected cells to mock, G129R and G129E transfected cells.

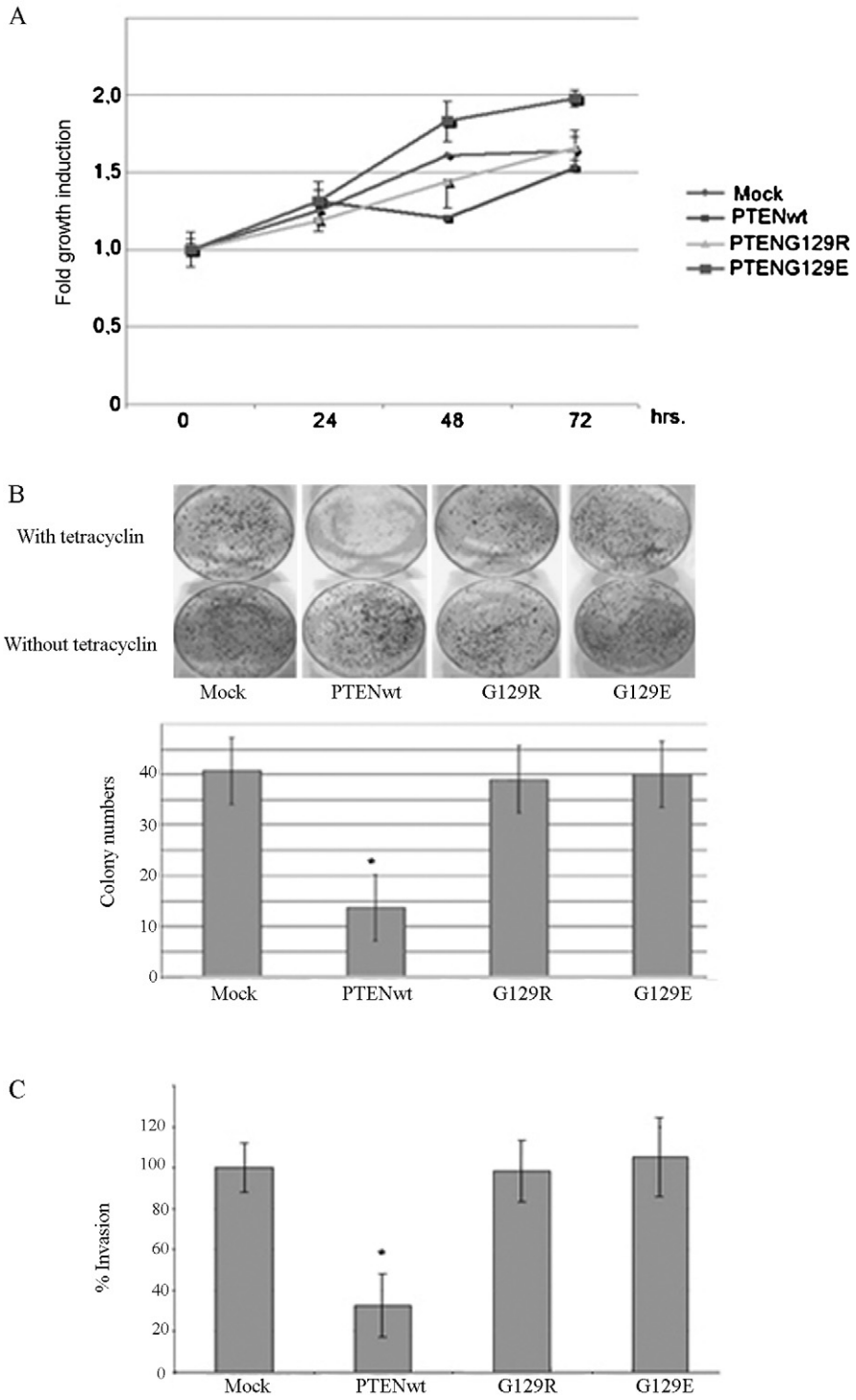

Fig. 3. The effects of ectopicly expressed PTEN on anchorage dependent or independent growth and invasiveness of PC14 cells. Cell proliferation was determined with using luminometric method at 0, 24, 48,72 time points (A). To determine the effects of PTEN-WT, PTEN-G129R and PTEN-G129E expression on anchorage independent growth, cells transfected with above plasmid were treated with 2 μg/ml tetracycline, after 3 weeks developed colonies were methanol fixed, stained with toluidine blue, and visible colonies were counted (B). The effects of PTENwt, G129R and G129E expression on PC14 cells invasion. 1×10^4 PC14 cells were seeded in to growth factor reduced matrigel invasion chamber in serum-free medium which included 2 μg/ml tetracycline. Chemoattractant conditions were created by addition of 10% FCS containing RPMI-1640 medium. After 24 h invading cells were fixed, stained and counted. Error bars indicate S.D., $^*p < 0.05$ comparing PTENwt transfected cells to mock, G129R and G129E transfected cells.

3.2. Effects of PTEN expression on anchorage independent growth and invasiveness of lung cancer cells

We next examined the biological effects of PTEN-WT, PTEN-G129E and PTEN-G129R on PC14 cells. As shown in Fig. 3A, there was no significant difference on the growth rate between the mock transfectants and cells expressing PTEN-WT, PTEN-G129E or PTEN-G129R under standard culture conditions. Since ability of cells to grow independently of adhesion is a feature of cancer cells, we examined the effect of PTEN expression on the anchorage independent growth of PC14 cells by a soft agar colony formation assay. As shown in Fig. 3B, tetracycline-induced expression of wild type

PTEN inhibited the growth of PC14 cells in soft agar, but neither PTEN-G129E nor PTEN-G129R had such an effect on PC14 cells. Therefore, anchorage independent growth but not anchorage dependent growth was suppressed by wild-type PTEN expression in PC14 cells.

To further explore whether PTEN inhibits cell invasion, we next performed a cell invasion assay using PC14 transfectants. As shown in Fig. 3C, cell invasiveness was reduced by 72% in PC14 cells expressing wild-type PTEN compared to a mock transfectant. Such invasiveness was not suppressed in cells expressing PTEN-G129E and PTEN-G129R, suggesting that inhibition of invasion was dependent on lipid phosphatase activity of PTEN. To confirm our results and to discard cell specific findings PTEN-negative PC9 and PC3 cells were transiently transfected with PTEN-WT, PTEN-G129R and PTEN-G129E. We observed that ectopic expression of PTEN can suppress PC9 and PC3 cell invasion by inhibition of AKT/NFkB pathway through its lipid phosphatase activity (Fig. 5).

3.3. *Effects of PTEN expression on invasiveness of mouse NIH3T3 cells*

Since the AKT activity was robustly affected by PTEN activity in PC14, PC9 and PC3 cells, we assumed that regulation of cell invasiveness by PTEN might be dependent on AKT activity in cells. To confirm this assumption, NIH3T3/CA-AKT cells expressing constitutively active AKT [38] were assessed by a cell invasion assay. AKT is constitutively phosphorylated in NIH3T3/CA-AKT cells but not in parental NIH3T3 cells (Fig. 4A), and NFkB activation was shown by a reporter assay (Fig. 4B). Therefore, we compared the invasiveness of parental NIH3T3 cells and NIH3T3/CA-AKT cells by a cell invasion assay. As shown in Fig. 4C, NIH3T3/CA-AKT cells showed 7 times higher invasiveness in comparison with parental NIH3T3 cells. These results support our hypothesis that cell invasiveness is largely regulated by AKT and NFkB activities in all PC14, PC9, PC3 and NIH3T3 cells.

4. Discussion

Lung cancer is the most common cause of cancer death worldwide [39]. Metastases are the major cause of death from cancer; however, molecular mechanisms of lung cancer metastasis remain largely unclear. In normal lung tissues, high levels of PTEN expression were detected in 100% [40], while, in lung cancer cell lines, PTEN expression was lost in 44% and reduced in 29% [41]. Our results indicates that PTEN expression is lost in ~50% of NSCLC cells. This finding was in good agreement with previous findings, showing that high levels of PTEN expression were detected in normal lung tissue [40], while PTEN expression was lost in 44% and reduced in 29% of lung cancer cell lines [41]. Moreover, PTEN deletion was reported to occur in 2% of NSCLC cells [5]. Therefore, it is likely that deletion is not a common mechanism of loss of PTEN expression. Noro et al. [37] reported that the PTEN gene is not deleted but its promoter region is hypermethylated in PC14 cells. Moreover, they also reported that, following 5-aza-dC treatment, PTEN mRNA and protein was increased in PC14 cells. They also showed in the same article that the mechanism of loss of PTEN expression in PC9 cells is histone deacetylation [37]. Furthermore, Soria et al. [36] reported that PTEN promoter is hypermethylated in H1299 cells and PTEN mRNA was increased after 5-aza-dC treatment in this cell line. It was reported that overexpression of phosphorylated AKT and loss of PTEN expression are associated with poor prognosis of patients with NSCLC [40]. In our previously published study, levels of AKT phosphorylation were enhanced by EGF treatment in PC3, PC9, PC14 and H1299 cells without PTEN expression, but unchanged in II-18 and PC13 cells with PTEN expression [42]. This result further

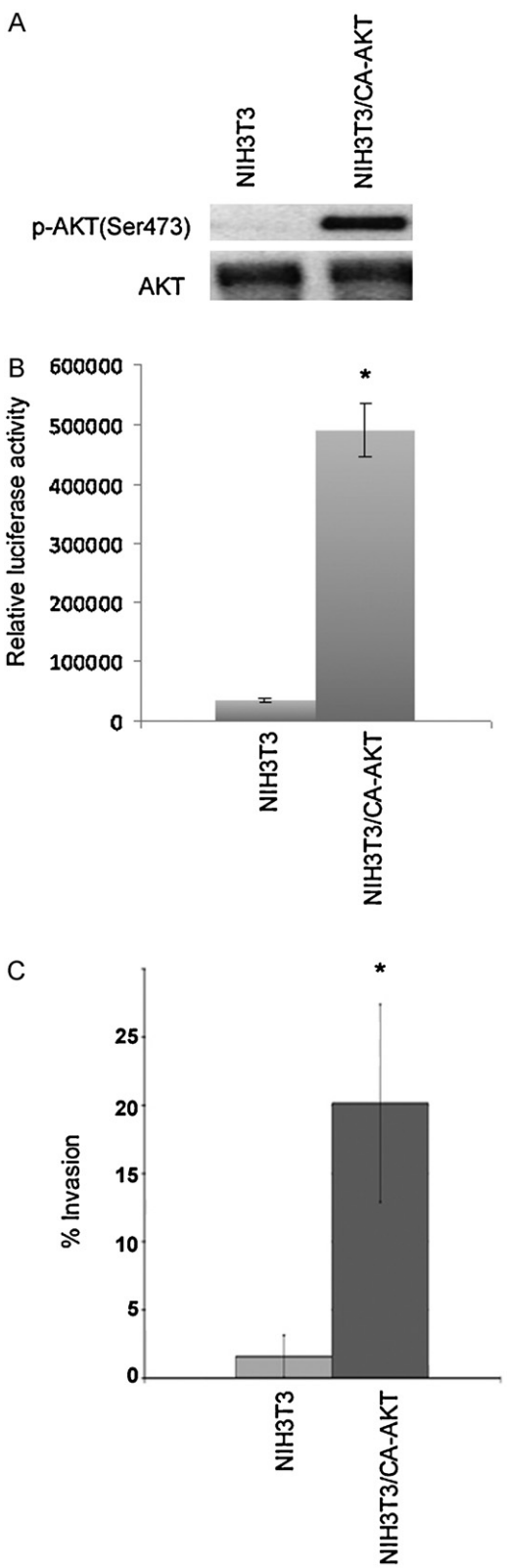

Fig. 4. The effects of AKT activation on NFkB activity and cellular invasiveness on NIH3T3 cells. We used NIH3T3 and CA-AKT expressing NIH3T3/CA-AKT cells for this experiment (A). To determine the effect of AKT activity on NFkB activation these cells were co-transfected with pGL3-NFkB-promoter-Luc (0.5 μg/well) and with pRL-*Renilla* (0.05 μg/well) vectors, 24 h after transfection cells were lysed and lusiferase activities were measured (B). Akt activation can induce cellular invasion in NIH3T3 cells (C). Error bars indicate S.D., $^{*}p < 0.005$ comparing NIH3T3/CA-AKT and NIH3T3 cells.

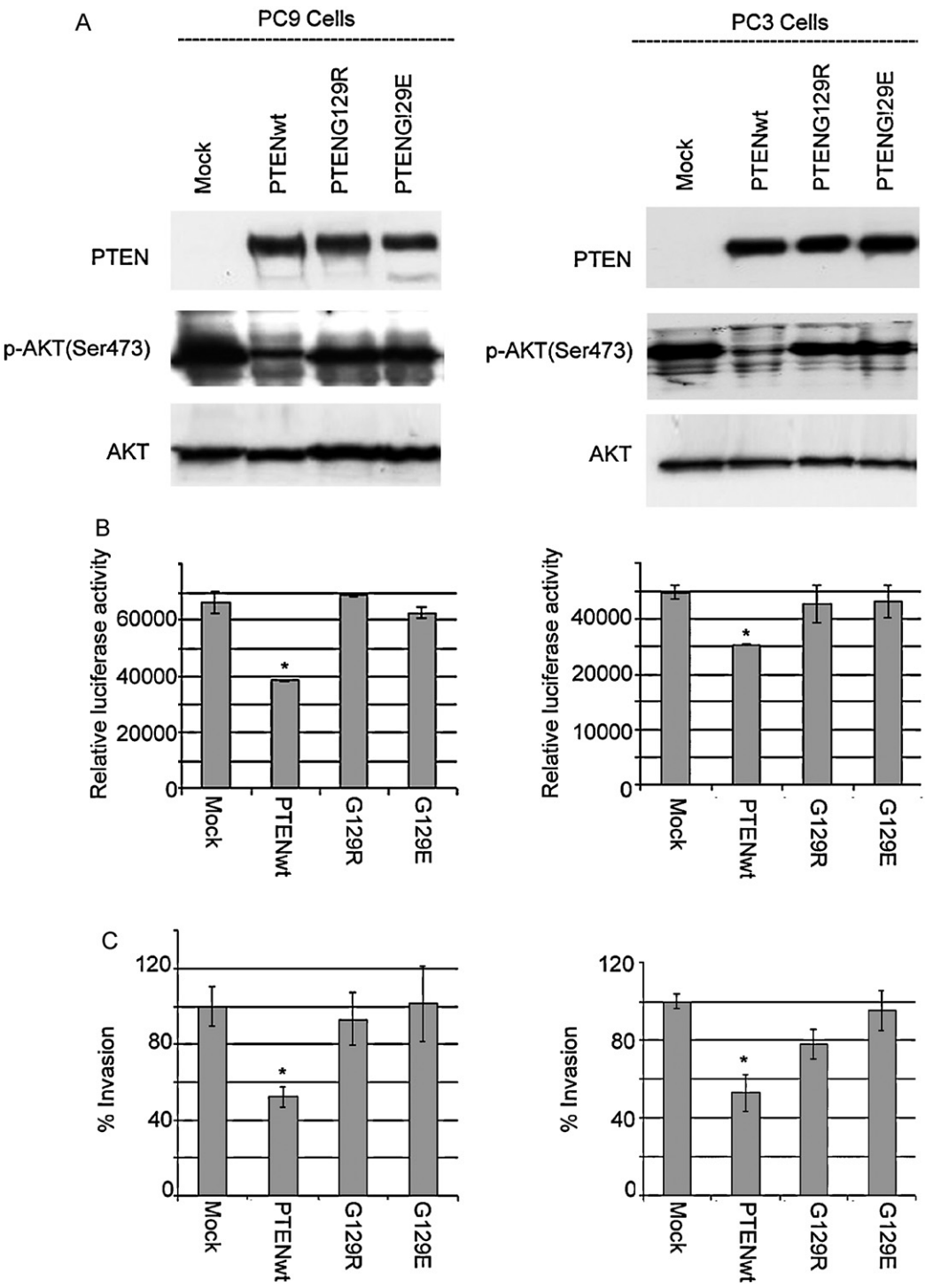

Fig. 5. Ectopic expression of PTEN and its effects on AKT phosphorylation, NFkB activation and cellular invasion on PC9 and PC3 cells. PC9 and PC3 cells were transiently transfected with pcDNA-PTENwt, pcDNA-PTEN-G129R, pcDNA-PTEN-G129E expression vectors by FUGEN HD transfection reagent. After 24 h of transfection PTEN expressions and their effects on AKT phosphorylations were determined by western blot anaysis (A). PC9 and PC3 cells were co-transfected with PTENwt, PTEN-G129R and PTEN-G129E expression vectors and with pRL-*Renilla* (0.05 μg/well) vectors and 24 h after transfection cells were lysed and luciferase activities were measured (B). Cells were transiently transfected with pcDNA-PTENwt, pcDNA-PTEN-G129R, pcDNA-PTEN-G129E expression vectors by FUGEN HD transfection reagent. After 24 h cells were counted and 1×10^4 cells were seeded in to growth factor-reduced invasion chamber in serum-free medium containing 2 μg/ml tetracycline. Chemoattractant conditions were created by addition of RPMI-1640 medium containing 10% FCS, after 24 h invasive cells were fixed, stained and counted (C). Error bars indicate S.D., $^*p < 0.05$ comparing PTENwt transfected cells to mock, G129R and G129E transfected cells.

supports the importance of PTEN expression in the regulation of AKT phosphorylation in lung cancer cells. Therefore, we assumed that PTEN inactivation and its effect on the PI3K/AKT/NFkB pathway could be important in lung cancer cell invasion. To address our hypothesis, we first examined the expression status of PTEN protein in 9 lung cancer cell lines. PTEN expression was lost in 5 and reduced in 4 of the 9 cell lines when compared with an immortalized small airway epithelial cell line. These results are consistent with previous results [40]. We also found increased AKT activities in cell lines with loss of PTEN expression. Therefore, AKT activ-

ity is well associated with the expression of PTEN in these 9 cell lines.

We then pursued the effect of PTEN expression on anchorage dependent and independent growth of PC14 cells. We first determined the *in vitro* growth rates of mock, PTEN-WT, PTEN-G129R and PTEN-G129E expressing PC14 cells, and found that all cells have similar proliferation rates, suggesting that PTEN activity does not affect PC14 cell proliferation under standard culture conditions. Furnari et al. [43] reported that PTEN-G129R and PTEN-G129E expression did not change the glioblastoma cell proliferation rates but PTEN-WT expression inhibited proliferation by the 7th day. Our results are not consistent with their findings; however, we determined the rate of proliferation only after 3 days. We next examined the effects of wild-type and mutant PTEN expression on anchorage independent growth. Only wild-type PTEN expression inhibited anchorage independent growth but neither catalytically inactive nor lipid phosphatase dead PTEN had an effect to inhibit the growth. Previous studies also showed that PTEN expression inhibits the anchorage independent growth of hepatoma [44], breast cancer and melanoma cell lines [45].

PTEN expression has been also shown to suppress invasion of breast cancer, glioma and colon cancer cells [46–48]. Therefore, we next attempted to elucidate whether PTEN inactivation is associated with invasion of lung cancer cells. We used PC14, PC9 and PC3 cells, which lack endogenous PTEN expression, for this experiment. We stably transfected PC14 cells and transiently transfected PC9 and PC3 cells with mock, PTEN-WT, PTEN-G129E, and PTEN-G129R. PTEN-WT inhibited the invasion of PC14, PC9 and PC3 cells respectively 72%, 48% and 46%, but mutants of PTEN did not, suggesting that lipid phosphatase activity of PTEN is essential for NSCLC cell invasion. PTEN can suppress prostate cancer cell [49] and glioma cell [47] invasion, but the mechanism of this suppression is different between prostate cancer cells and glioma cells. Shukla et al. [49] reported that PTEN can suppress prostate cancer cell invasion through the suppression of the PI3K/AKT pathway by its lipid phosphatase activity, however, Maier et al. [47] reported that the PTEN lipid phosphatase domain is not required for invasion of glioma cells. Our results clearly indicate that PTEN suppresses lung cancer cell invasion through its lipid phosphatase activity. Recently, Zhang et al. [50] reported that microRNA-21 promotes invasion of lung cancer cells by repressing expression of PTEN, however, they didn't elucidate the molecular mechanisms of PTEN-mediated inhibition of lung cancer cell invasion. Since NFkB is a major target of activated AKT, we attempted to find interactions between the AKT/NFkB activity and lung cancer cell invasion. Therefore, reporter vectors for NFkB luciferase and renilla luciferase were co-transfected in mock, PTEN-WT, PTEN-G129R and PTEN-G129E expressing PC14 cells. The result indicated that only wild-type PTEN inhibits NFkB activity, and implies that PTEN inhibits NFkB activity through its lipid phosphatase activity and PTEN can inhibit lung cancer cell invasion through the suppression of the PI3K/AKT/NFkB pathway. To further confirm our hypothesis, we used parental NIH3T3 and NIH3T3CA-AKT cells, in which constitutively active AKT is overexpressed [38]. AKT activation induced NFkB activation and cellular invasion in NIH3T3 cells, supporting that AKT-mediated NFkB activation is necessary for lung cancer cell invasion.

In summary, we report here for the first time that activation of the AKT/NFkB pathway is necessary for the anchorage independent growth and invasion of lung cancer cells, and PTEN expression can suppress both processes by inhibiting the AKT/NFkB pathway.

Conflict of interest statement

The authors declare no conflict of interest for this article.

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References

[1] Wang SI, Puc J, Li J, Bruce JN, Cairns P, Sidransky D. Somatic mutations of PTEN in glioblastoma multiforme. Cancer Res 1997;19:4183–6.
[2] Guldberg P, Thor Straten P, Birck A, Ahrenkiel V, Kirkin AF, Zeuthen J. Disruption of the MMAC1/PTEN gene by deletion or mutation is a frequent event in malignant melanoma. Cancer Res 1997;17:3660–3.
[3] Cairns P, Okami K, Halachmi S, Halachmi N, Esteller M, Herman JG. Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. Cancer Res 1997;22:4997–5000.
[4] Rhei E, Kang L, Bogomolniy F, Federici MG, Borgen PI, Boyd J. Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinomas. Cancer Res 1997;17:3657–9.
[5] Kohno T, Takahashi M, Manda R, Yokota J. Inactivation of the PTEN/MMAC1/TEP1 gene in human lung cancers. Genes Chromosome Cancer 1998;22:152–6.
[6] Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, et al. Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. Cancer Res 1997;18:3935–40.
[7] Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, et al. Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat Genet 1997;11:64–7.
[8] Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, et al. Germline mutations in PTEN are present in Bannayan-Zonana syndrome. Nat Genet 1997;4:333–4.
[9] Pilarski R, Eng C. will the real Cowden syndrome please stand up (again?) expanding mutational and clinical spectra of the PTEN hemartoma tumor syndrome. J Med Genet 2004;41:323–6.
[10] Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. Pten is essential for embryonic development and tumour suppression. Nat Genet 1998;19: 348–55.
[11] Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, del Barco Barrantes I, et al. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr Biol 1998;22:1169–78.
[12] Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, et al. PTEN a putative protein tyrosine phosphatase gene mutated in human brain, breast and prostate cancer. Science 1997;275:1943–7.
[13] Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, et al. Identification of a candidate tumor suppressor gene MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Natl Genet 1997;15:356–62.
[14] Li DM, Sun H. TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. Cancer Res 1997;11:2124–9.
[15] Maehama T, Dixon JE. The tumor suppressor PTEN/MMAC1 dephosphorylates the lipid secondary messenger, phosphatidylinositol 3,4,5-triphosphate. J Biol Chem 1998;273:13375–8.
[16] Auger KR, Serunian LA, Soltoff SP, Libby P, Cantley LC. PDGF dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell 1989;57:167–75.
[17] Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, et al. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. Proc Natl Acad Sci U S A 1998;95:13513–8.
[18] Haas-Kogan D, Shalev N, Wong M, Mills G, Yount G, Stokoe D. Protein kinase B (PKB/Akt) activity is elevated in glioblastoma cells due to mutation of the tumor suppressor PTEN. Curr Biol 1998;8:1195–8.
[19] Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, et al. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997;275:661–5.
[20] Datta SR, Dudek H, Tao H, Masters S, Fu H, Gotoh Y, et al. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 1997;2:231–41.
[21] Dan HC, Baldwin AS. Differential involvement of IkappaB kinases alpha and beta in cytokine- and insulin-induced mammalian target of rapamycin activation determined by Akt. J Immunol 2008;11:7582–9.
[22] Downward J. Ras signaling and apoptosis. Curr Opin Genet Dev 1998;8:49–54.
[23] Ahmed NN, Grimes HL, Bellacosa A, Chan TO, Tsichlis PN. Transduction of inteleukin-2 antiapoptotic and proliferative signals via AKT protein kinase. Proc Natl Acad Sci U S A 1997;94:3627–32.
[24] Datta K, Franke TF, Chan TO, Makris A, Yang SI, Kaplan DR, et al. AH/PH domain mediated interaction between AKT molecules and its potential role in AKT regulation. Mol Cell Biol 1995;15:2304–10.
[25] Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS. AKT promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 1999;98:857–68.

[26] Kennedy SG, Kandel ES, Cross TK, Hay N. Akt/Protein kinase B inhibits cell death by preventing the release of cytochrome c from mitochondria. Mol Cell Biol 1999;19:5800–10.
[27] Brodt P, Samani A, Navab R. Inhibition of the type I insulin-like growth factor receptor expression and signaling: novel strategies for antimetastatic therapy. Biochem Pharmacol 2000;60:1101–7.
[28] Vivanco I, Sawyers CL. The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2002;2:489–501.
[29] Jiang BH, Jiang G, Zheng JZ, Lu Z, Hunter T, Vogt PK. Phosphatidylinositol 3-kinase signaling controls levels of hypoxia-inducible factor 1. Cell Growth Differ 2001;12:363–9.
[30] Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of world-wide burden of cancer in 2008. GLOBOCAN 2008. Int J Cancer June 17, 2010, 17 [Epub ahead of print].
[31] Nalate RB, Zaretsky SL. ZD1839 (Iressa): what's in it fort he patients. Oncologist 2002;7(4):25–30.
[32] Woodhouse EC, Chuaqui RF, Liotta LA. General mechanisms of metastasis. Cancer 1997;80:1529–37.
[33] Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, et al. PTEN, the tumor suppressor from human chromosome10q23, is a dual-specificity phosphatase. Proc Natl Acad Sci U S A 1997;94:9052–7.
[34] Blaskovich MA, Lin Q, Delarue FL, Sun J, Park HS, Coppola D, et al. A platelet-derived growth factor binding molecule with antiangiogenic and anticancer activity against human tumors in mice. Nat Biotechnol 2000;18:1065–70.
[35] Roy BC, Kohno T, Iwakawa R, Moriguchi T, Kiyono T, Morishita K, et al. Involvement of LKB1 in epithelial-mesenchymal transition (EMT) of human lung cancer cells. Lung Cancer 2010;70(2):136–45.
[36] Soria JC, Lee HY, Lee JI, Wang L, Issa JP, Kemp BL, et al. Lack of PTEN expression in non-small cell lung cancer could be related to promoter methylation. Clin Cancer Res 2002 May;8(5):1178–84.
[37] Noro R, Gemma A, Miyanaga A, Kosaihira S, Minegishi Y, Nara M. PTEN inactivation in lung cancer cells and the effect of its recovery on treatment with epidermal growth factor receptor tyrosine kinase inhibitors. Int J Oncol 2007;31(5):1157–63.
[38] Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, et al. Phosphatidylinositol 3-kinase/AKT/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci U S A 2001;98:4640–5.
[39] Greenlee RT, Hill-Harmon MB, Murray T, Thun M. Cancer statistics. CA Cancer J Clin 2001;51:16–36.
[40] Tang JM, He QY, Guo RX, Chang XJ. Phosphorylated AKT overexpression and loss of PTEN expression in non-small lung cancer confers poor prognosis. Lung Cancer 2006;51:181–91.
[41] Marsit CJ, Zheng S, Aldape K, Hinds PW, Nelson HH, Wiencke JK, et al. PTEN expression in non-small-cell lung cancer: evaluating its relation to tumor characteristics allelic loss, and epigenetic alteration. Human Pathol 2005;36:768–76.
[42] Akca H, Tani M, Hishida T, Matsumoto S, Yokota J. Activation of the AKT and STAT3 pathways and prolonged survival by a mutant EGFR in human lung cancer cells. Lung Cancer 2006;54:25–33.
[43] Furnari FB, Lin H, Huang HS, Cavenee WK. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. Proc Natl Acad Sci U S A 1997;94:12479–84.
[44] Nakanishi K, Sakamoto M, Yasuda J, Takamura M, Fujita N, Tsuruo T, et al. Critical involvement of the phosphatidylinositol 3-kinase/Akt pathway in anchorage-independent growth and hematogeneous intrahepatic metastasis of liver cancer. Cancer Res 2002;10:2971–5.
[45] Lobo GP, Waite KA, Planchon SM, Romigh T, Nassif NT, Eng C. Germline and somatic cancer-associated mutations in the ATP-binding motifs of PTEN influence its subcellular localization and tumor suppressive function. Hum Mol Genet 2009;15:2851–62.
[46] Heering J, Erlmann P, Olayioye MA. Simultaneous loss of the DLC1 and PTEN tumor suppressors enhances breast cancer cell migration. Exp Cell Res 2009;315:2505–14.
[47] Maier D, Jones G, Li X, Schönthal AH, Gratzl O, Van Meir EG, et al. The PTEN lipid phosphatase domain is not required to inhibit invasion of glioma cells. Cancer Res 1999;59:5479–82.
[48] Bowen KA, Doan HQ, Zhou BP, Wang Q, Zhou Y, Rychahou PG, et al. PTEN loss induces epithelial–mesenchymal transition in human colon cancer cells. Anticancer Res 2009;11:4439–49.
[49] Shukla S, Maclennan GT, Hartman DJ, Fu P, Resnick MI, Gupta S. Activation of PI3K-Akt signaling pathway promotes prostate cancer cell invasion. Int J Cancer 2007;121:1424–32.
[50] Zhang JG, Wang JJ, Zhao F, Liu Q, Jiang K, Yang GH. MicroRNA-21 represses tumor suppressor PTEN and promotes growth and invasion in non-small cell lung cancer (NSCLC). Clin Chim Acta 2010;411:846–52.