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Frequent activation of AKT in non-small cell lung carcinomas and preneoplastic bronchial lesions

Binaifer R.Balsara[1,*], Jianming Pei[1,*], Yasuhiro Mitsuuchi[1], Robert Page[2], Andres Klein-Szanto[2], Hao Wang[3], Michael Unger[4] and Joseph R.Testa[1,5]

[1]Cancer Genetics, [2]Tumor Cell Biology, [3]Biostatistics and [4]Pulmonary Cancer Detection and Prevention Programs, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

[5]To whom correspondence should be addressed. Tel: +1 215 728 2610; Fax: +1 215 214 1623;
Email: joseph.testa@fccc.edu

AKT is frequently activated in various cancers, but its involvement in lung tumor development and progression is not well established. We examined AKT activity by immunohistochemistry in 110 non-small cell lung carcinomas (NSCLCs) using tissue microarrays. AKT activation was observed in 56 (51%) tumors. To further validate activation of the AKT pathway in this series, we examined the phosphorylation status of the mammalian target of rapamycin (mTOR) and forkhead (FKHR), two downstream targets of AKT. Positive staining for phospho-mTOR and phospho-FKHR were detected in 74% and 68% of tumors, respectively, and was significantly associated with activation of AKT. Tumors positive for phosphorylated (active) AKT were present with a similar frequency in low stage (I/II) and high stage (III/IV) tumors, raising the possibility that AKT activation occurs early in tumor progression. We therefore examined AKT activity in 25 bronchial epithelial lesions from 12 patients at high risk of lung cancer. Metaplastic/dysplastic areas showed AKT activity, whereas normal and hyperplastic bronchial epithelia exhibited little or no activity. Since some bronchial epithelial lesions may develop into invasive cancers, we examined the effect of AKT on invasiveness of lung cancer cells, using an *in vitro* cell invasion assay. Transfection of NSCLC cells with wild-type AKT increased invasiveness in response to hepatocyte growth factor, whereas transfection with dominant negative AKT abrogated this effect. Collectively, these data suggest that AKT activation is a frequent and early event in lung tumorigenesis, which may enhance risk of progression to malignancy. Thus, AKT represents a potentially important target for chemoprevention in individuals at high risk of NSCLC.

Abbreviations: ADC, adenocarcinoma; DAPI, diamidino-2-phenylindole; FKHR, forkhead; GFP, green fluorescent protein; HGF, hepatocyte growth factor; LIFE, lung imaging fluorescence endoscopy; mTOR, mammalian target of rapamycin; NSCLC, non-small cell lung carcinoma; p-AKT, phosphorylated AKT; PI3K, phosphatidylinositol-3 kinase; p-FKHR, phosphorylated FKHR; p-mTOR, phosphorylated mTOR; SCC, squamous cell carcinoma; TMA, tissue microarray.

*These authors contributed equally to this work.

Introduction

AKT/protein kinase B is a major downstream target of growth factor receptor tyrosine kinases that signal via phosphatidylinositol-3 kinase (PI3K) (1–4). The AKT kinase family consists of several closely related, highly conserved cellular homologs designated *AKT1*, *AKT2* and *AKT3* (reviewed in 5). Binding to PI3K phospholipid products triggers the translocation of AKT kinases to the plasma membrane. Upon membrane localization, AKT is phosphorylated at Thr308/309 in the kinase activation loop and Ser473/474 in the C-terminal tail. Phosphorylation on these residues is induced by growth factor stimulation and inhibited by the specific PI3K inhibitor LY294002.

Mounting evidence suggests that AKT perturbations play an important role in human malignancy (5). Recent investigations have documented frequent activation of AKT kinases in a variety of tumor types, such as ovarian, breast, prostate and pancreatic carcinomas (6–8). Receptor-mediated activation of PI3K or loss of expression of the phosphatase PTEN, one of the most frequently mutated tumor suppressors, results in elevated AKT activity. AKT signaling appears to play a prominent role in several processes thought to be critical in tumorigenesis, including aberrant cell proliferation, inhibition of programmed cell death, telomerase activation and promotion of angiogenesis and tumor cell invasiveness (reviewed in 5).

Lung cancer has a dismal prognosis, which has been attributed largely to ineffective early detection methods and lack of successful therapeutic options for metastatic disease (9). This has spurred efforts to identify biomarkers for early detection as well as the development of molecularly targeted therapies. Recent evidence suggests that AKT contributes to resistance to chemotherapy, radiation and tyrosine kinase inhibitors by mediating survival signals that protect cells from undergoing apoptosis (10–13). For example, treatment of non-small cell lung carcinoma (NSCLC) cell lines with LY294002 greatly potentiated chemotherapy- and radiation-induced apoptosis in cells with constitutive activation of AKT, but did not significantly increase therapy-induced apoptosis in cells with low basal levels of AKT (10). Thus, inhibitors of the AKT pathway may be effective, in combination with other anticancer drugs, for the treatment of tumors with activated AKT signaling (12). Moreover, if AKT activation were found to be an early event in lung tumorigenesis, it could serve as a useful target for chemoprevention with an inhibitor of the PI3K/AKT pathway.

Growth factors and their receptors play an important role in the development and/or maintenance of the malignant phenotype of NSCLC and participate in autocrine/paracrine growth stimulation (14). NSCLCs overexpress a range of growth factors and their receptors. Some of the growth factor receptors implicated in NSCLCs include hepatocyte growth factor (HGF), epidermal growth factor, platelet derived growth factor and insulin-like growth factor receptors (15). Such

mitogen-activated receptor tyrosine kinases have been linked to the activation of AKT through PI3K (1,16,17). We therefore investigated a series of NSCLCs to evaluate AKT activity in this tumor type. AKT affects numerous downstream targets either directly or indirectly, many of which are involved in cell survival or protein translation. The forkhead (FKHR) transcription factors fall into the first category, whereas the mammalian target of rapamycin (mTOR) is involved in translation regulation (18). Here we provide evidence indicating that activation of AKT is a frequent and early event in lung tumorigenesis, resulting in phosphorylation of these downstream targets. We also demonstrate that AKT activation increases invasiveness of NSCLC cells.

Materials and methods

Tissue specimens and cell lines

All primary tumor specimens were selected randomly from patients who underwent surgery at the Fox Chase Cancer Center between 1993 and 1999. Sixty-six adenocarcinomas (ADCs), 44 squamous cell carcinomas (SCCs) and 27 normal lung specimens were included in the study. Twenty-five bronchial epithelial lesions were obtained by lung imaging fluorescence endoscopy (LIFE) from 12 individuals with a long history of smoking, some of whom were at high risk of recurrence of NSCLC. Demographic characteristics of the samples are shown in Table I. Cell lines used for *in vitro* assays were established from primary NSCLCs as described earlier (19).

Tissue microarray

Tissue microarrays (TMAs) were made from archived samples from the Tumor Bank Facility of the Fox Chase Cancer Center. They were constructed by harvesting 1 mm tissue cores from paraffin embedded samples. The punches of tissue cores were arrayed in 15 × 15 mm paraffin blocks. Sections, 5 μm in thickness, were cut and mounted on adhesive-coated slides and stored in a dry environment until use.

Immunohistochemical staining

Tissue sections were deparaffinized in xylene, rehydrated through graded solutions of alcohol and subjected to specific antibody staining for various antigens using a Biotek Techmate 1000 robotic immunostainer (Ventana Medical Systems, Tuscon, AZ). For antigen retrieval, slides were boiled in 10 mM citrate buffer, pH 6.0, for 10 min using a 750 W microwave oven. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 for 20 min and sections were washed and blocked with serum for 30 min. All antibodies and phosphopeptides used for immunohistochemistry were obtained from Cell Signaling Technologies (Beverly, MA). Pan-phospho-AKT (Ser473) antibody was used to detect phosphorylated AKT (p-AKT). The specificity of the antibody was verified by immunostaining following preincubation of the antibody with a phosphopeptide to AKT Ser473, at a 1:10 ratio for 2 h, as per the manufacturer's specifications. Phospho-mTOR (Ser2448) and phospho-FKHR (Ser256) were used to detect the phosphorylated forms of m-TOR (p-mTOR) and FKHR (p-FKHR), respectively. The specificities of these antibodies were also confirmed by preabsorption with the respective phosphopeptide, at a ratio of 1:1 for 0.5 h, in accordance with the manufacturer's recommendations. Antigen–antibody complexes were detected by the avidin–biotin–peroxidase method using 3,3′-diaminobenzidine (BioGenex, San Ramon, CA) and counterstained with hematoxylin. The surrounding non-neoplastic stroma served as an internal negative control for each slide. Each tumor specimen was arrayed in duplicate and a semi-quantitative scoring system was used for evaluation. Since all of the arrayed tumors had a high percentage of tumor cells that stained positively for p-AKT, p-mTOR and p-FKHR, the scoring system was based primarily on the staining intensity: +, mild cytoplasmic stain in ~40–50% of cells which is clearly more intense than the marginal stain seen in the stroma; ++, moderate stain which is intermediate between + and +++; +++, intense dark brown cytoplasmic stain in 50–85% of cells. Two examples are shown in Figure 1C.

Table I. Demographic characteristics of 110 NSCLCs

Characteristic	Number (%)
Age distribution (years)	
<50	4 (4%)
50–59	26 (24%)
60–69	42 (38%)
≥70	38 (35%)
Gender	
Male	65 (59%)
Female	45 (41%)
Smoking status[a]	
Smoker or former smoker	80 (73%)
Non-smoker	9 (8%)

[a]Smoking status was not available for 21 cases.

Statistical analysis

Fisher's exact test was used to examine the association of p-AKT with clinical characteristics such as histological type, tumor stage and tumor grade. A log rank test was used to compare survival status between p-AKT-positive and -negative tumors. Correlation of p-AKT levels with p-mTOR and p-FKHR status was assessed using the Jonckheere–Terpstra test. All statistical computations were carried out using SAS software.

Western blot analysis

To detect AKT2 expression, 200 μg cell lysate was immunoprecipitated with 2 μg anti-AKT2 antibody (Upstate Biotechnology, Lake Placid, NY), followed by incubation with protein A:protein G agarose beads. Immunoprecipitates were subjected to 7.5% SDS–PAGE. Membranes were probed with AKT2 (d-17) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For Met expression, cell lysates were subjected to SDS–PAGE and Met (C-12) (Santa Cruz Biotechnology) was used as the primary antibody. To detect expression of AKT2 following transfection of 110-T cells, an anti-HA antibody (BAbCO, Richmond, CA) was used. Detection of antigen bound antibody was carried out with a Renaissance Chemiluminescence Reagent Plus system (NEN Life Sciences, Boston, MA).

In vitro protein kinase assay

In vitro protein kinase assays were performed as described (17) with minor modifications. In brief, AKT2 was immunoprecipitated from 600 μg total tissue lysate using the specific antibody with a mixture of protein A:protein G agarose beads. The immunocomplexes were extensively washed with lysis buffer and subsequently equilibrated with kinase reaction buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol and 1 mM EDTA). The kinase assays were carried out at room temperature for 30 min in the presence of 5 μM [γ-^{32}P]ATP (300 GBq/mmol) using histone H2B as a substrate. The reactions were terminated by the addition of 2× Laemmli sample loading buffer and then subjected to SDS–PAGE. The amount of radioactivity incorporated was visualized by autoradiography. Detection of AKT2 activity in transfected cells was assessed by kinase assay after immunoprecipitation with anti-HA antibody.

Transfection with plasmid constructs

110-T cells were grown in 6-well plates and then transiently transfected with 2 μg/ml plasmid DNA to express green fluorescent protein (GFP) expression vector alone as a control, GFP-HA-AKT2 (wild-type) or GFP-HA-AKT2e299k (an inactive form of AKT2 due to a point mutation of Glu299 to Lys) (17). Fugene 6 (Roche Molecular Biochemicals, Indianapolis, IN) was used as a transfection reagent according to the manufacturer's instructions.

In vitro cell invasion assay

The chemoinvasion assay was performed using BioCoat growth factor reduced matrigel invasion chambers (Beckton Dickinson, Bedford, MA) according to the manufacturer's suggestions. Briefly, NSCLC cell lines 110-T and H125-T (19) were trypsinized, washed and 0.5 × 10^5 cells/500 μl of Dulbecco's modified Eagle's medium with 0.1% bovine serum albumin were plated on the inserts. Serum or 15 U/ml HGF was used as a chemoattractant. After 24 h incubation, the invading cells on the lower surface of the inserts were fixed with 3.5% paraformaldehyde for 10 min, washed with phosphate-buffered saline and counterstained with the nuclear stain diamidino-2-phenylindole (DAPI) for 5 min. The data are expressed as the ratio of invading cells observed in response to serum or HGF compared with that in control, serum-starved cells. An invasion assay using 110-T cells transiently transfected with the plasmid constructs was carried out in a similar fashion. HGF (15 U/ml) was used as a chemoattractant. Invasion was assessed after 24 h. Inserts were fixed and counterstained as described earlier. The GFP and DAPI images were captured using a cooled CCD camera (Photometrics, Tucson, AZ) and analyzed with Quips software (Vysis, Downers Grove, IL). Four or five fields per insert were photographed and counted for each assay. The percentage of

GFP-positive cells invaded was used as a measure of invasion after normalizing for the transfection efficiency of that particular plasmid clone. Finally, the values were plotted as percent cells invaded relative to vector. Each determination shown represents the average of three separate experiments.

Results

AKT activity by immunohistochemistry

We evaluated AKT activity in 110 NSCLCs and 27 normal lung samples using TMAs. Among the entire tumor set, 56 of 110 NSCLCs (51%) stained positive (score of 1–3) for p-AKT, whereas the remaining tumor samples had weak or absent staining (score of 0–0.5). The available phospho-specific AKT antibody does not differentiate between the three isoforms of AKT, so our immunohistochemical analyses could not determine the frequency of activation of each AKT isoform. However, immunostaining revealed that all of our tumors expressed variable levels of AKT2. Tumors expressing p-AKT showed positive staining in the cytoplasm and sometimes in the nucleus, whereas the surrounding stromal cells exhibited little or no staining (Figure 1A). Twenty-three of 110 tumors (21%) had high AKT activity, with a score of 2–3. The clinical characteristics and the p-AKT status of the 110 NSCLCs are summarized in Table II. p-AKT expression was not associated with histological subtype ($P = 0.1779$) and did not correlate with survival ($P = 0.7964$). Median survival for all patients was 24 months and median follow-up time was 21.5 months. These characteristics were not significantly different between the p-AKT-positive and p-AKT-negative groups. For the p-AKT-positive cases the median survival and follow up times were 23 and 20.5 months, respectively. Similarly, for the p-AKT-negative cases the median survival

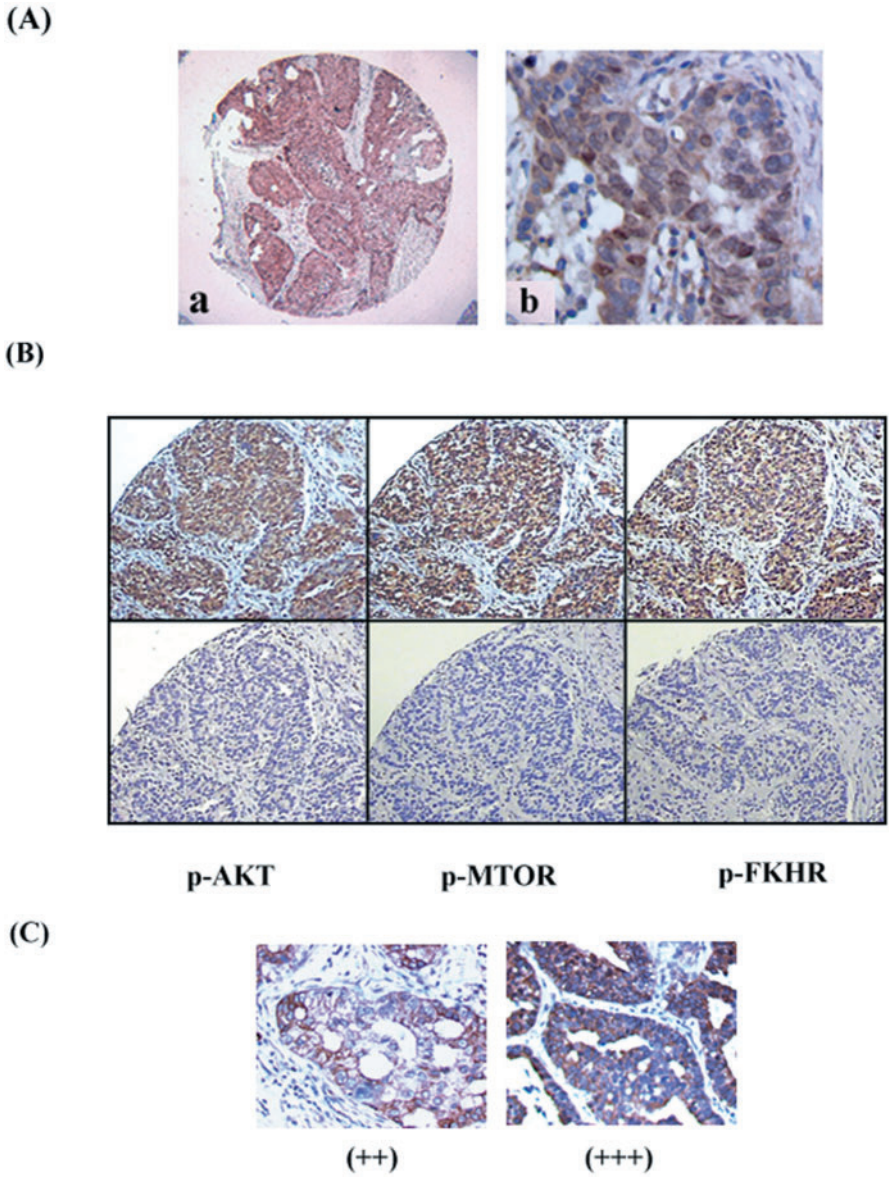

Fig. 1. (**A**) Positive immunohistochemical staining of NSCLC specimens. (**a**) TMA core of a lung SCC (×5 magnification). (**b**) Section from another SCC at higher magnification (×400). Note cytoplasmic and nuclear staining in most tumor cells. (**B**) (Upper) Adenocarcinoma of the lung showing good correlation between phospho-AKT, phospho-mTOR and phospho-FKHR immunostaining. (Lower) Reduced staining after preabsorbing the antibodies with the respective peptides. ×20 magnification. (**C**) Two adenocarcinomas of the lung showing moderate (++) and intense (+++) staining to illustrate examples for scoring.

and follow up times were 26 and 21 months, respectively. The frequency of p-AKT-positive cases was similar in low stage (I/II) and high stage (III/IV) tumors ($P = 0.4357$), as well as in low grade (well-differentiated or moderately differentiated) and high grade (poorly differentiated) tumors ($P > 0.3391$). This raised the possibility that AKT activation occurs early in tumor progression.

Activation of downstream targets mTOR and FKHR

To validate frequent activation of the AKT pathway in NSCLC, we examined the phosphorylation status of mTOR and FKHR by immunohistochemistry in our series of 110 NSCLCs. Positive staining for p-mTOR was detected in 74% of cases, with 52% showing moderate to intense staining (score 2–3). Positive staining for p-FKHR was observed in 68% of cases, with moderate to intense staining in 45% of cases. Activation of AKT correlated well with phosphorylation of these downstream markers (see, for example, Figure 1B). In 74% of the tumors, p-AKT and p-mTOR staining showed a similar trend, and a statistically significant association between the two was observed ($P < 0.0001$, Jonckheere–Terpstra trend test), consistent with prior experimental evidence demonstrating involvement of translational control in oncogenic transformation by PI3K and AKT (18). Similarly, a correlation between p-AKT and p-FKHR staining was observed in 71% of tumors ($P < 0.0001$).

Table II. Clinical features and p-AKT status in 110 NSCLCs

	ADC	SCC	AKT-positive	*P* value
Total	66	44	56 (51%)	0.1779
Stage[a]				
I/II	36	28	34 (53%)	0.4357
III/IV	28	16	21 (48%)	
Grade[b]				
1	6	2	3 (38%)	
2	27	19	21 (46%)	0.3391
3	32	23	31 (56%)	

[a]Staging for two ADC cases not known.
[b]Grade for one case not known.

AKT activity in preinvasive lung lesions

To address the possibility that AKT activation is an early event in tumor progression, we evaluated AKT activity by immunohistochemistry in 25 preneoplastic and preinvasive bronchial lesions (Figure 2). Eight of the 25 lesions were confirmed to have areas indicative of mild to severe dysplasia, all of which were positive for p-AKT. Squamous metaplasia was seen in eight lesions, and these were also positive for p-AKT. Five dysplastic lesions and three metaplastic lesions had high (score of 2) AKT activity. Hyperplastic areas seen in nine biopsies were weakly positive for p-AKT. Areas with normal bronchial epithelium showed little or no staining for p-AKT. These data suggest that AKT activation may occur early in the pathogenesis of lung cancer.

Role of AKT in tumor cell invasiveness

The diverse biological effects of HGF are mediated through the phosphorylation of its receptor (Met) and subsequent activation of various signaling molecules, one being AKT (21). Five human NSCLC cell lines were characterized for AKT activity and Met expression. As shown in Figure 3A, all of the cell lines expressed AKT2, and AKT2 kinase activity correlated well with the expression levels in these cell lines. For invasion studies, two NSCLC cell lines, 110-T and H125-T, were examined in relation to the expression of Met. 110-T cells were found to express Met, while H125-T cells do not express this receptor (Figure 3A). Both cell lines showed cell invasion activity when serum was used as a chemoattractant

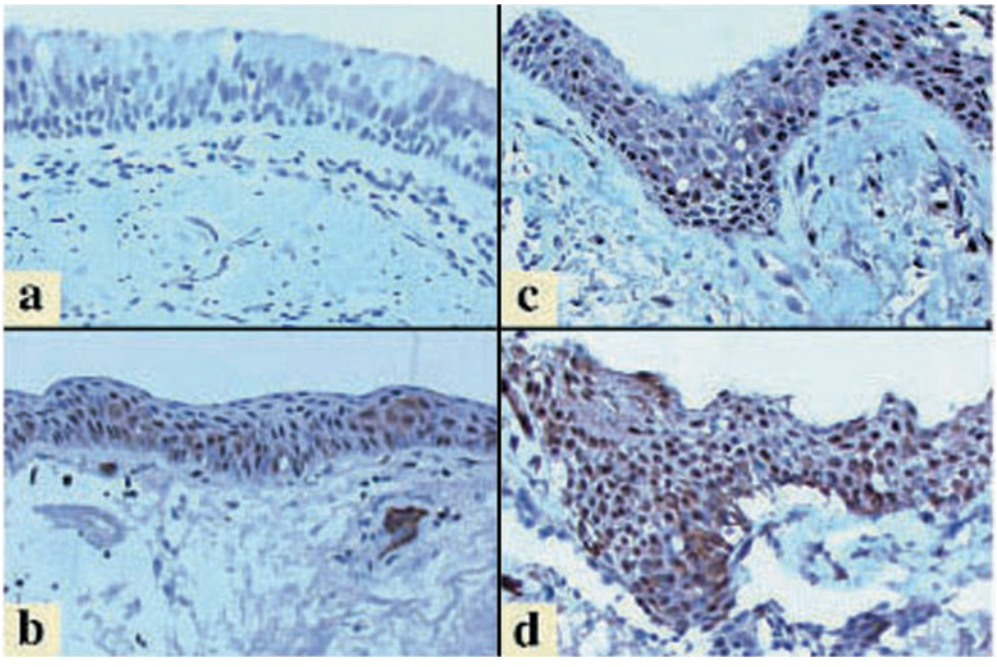

Fig. 2. Immunohistochemical staining of LIFE specimens with phospho-specific AKT antibody. (**a**) Lung specimen showing normal bronchial epithelium and absence of immunostaining. (**b**) Metaplastic bronchial epithelium staining positively for phospho-AKT. (**c** and **d**) Mild and moderate dysplasia of the bronchial epithelium showing moderate to intense immunostaining, indicative of AKT activation. ×200 magnification.

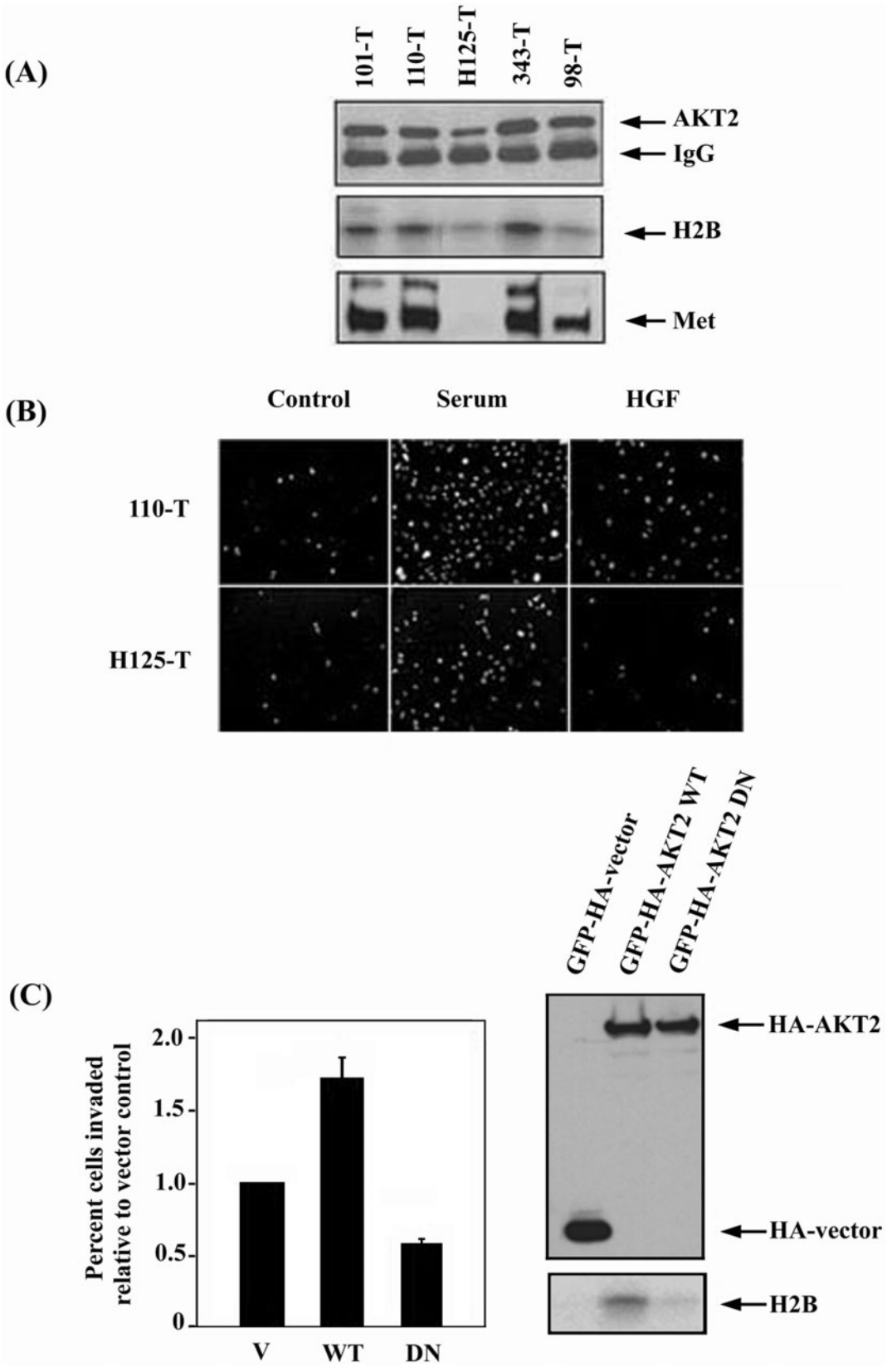

Fig. 3. (**A**) (Upper) AKT2 expression in human NSCLC cell lines. (Middle) Representative AKT2 *in vitro* kinase assay showing phosphorylation of histone H2B used as a substrate. (Lower) Met expression seen in four NSCLC cell lines. H125-T cells do not express the receptor. (**B**) HGF-dependent invasiveness in NSCLC cell lines. (Upper) 110-T cells, which express abundant Met, invade when serum or HGF is used as a chemoattractant. (Lower) H125-T cells, which do not express Met, invade in response to serum but not in response to HGF. (**C**) (Left) Bar graph showing increased invasiveness of the wild-type AKT2 (WT)-transfected 110-T cells compared with cells transfected with vector alone (V) or dominant negative AKT2 (DN). (Right upper) Western blot analysis to show identical expression levels of AKT2 in cells transfected with constructs for WT and DN AKT2. (Right lower) *In vitro* kinase assay showing additive baseline AKT2 activity in 110-T cells transfected with WT AKT2 construct, while DN AKT2 expression did not show any AKT2 activity.

(Figure 3B), with the average fold increase being 12.8 with 110-T cells and 11.3 with H125 cells. However, when HGF was used as a chemoattractant, the average fold increase was 7.2 for 110-T cells but only 1.1 for the Met receptor-negative H125-T cells (Figure 3A). To determine whether HGF-induced invasiveness in 110-T cells is associated with AKT activation, we transfected these cells with various AKT constructs. As shown in Figure 3B, cells transfected with wild-type AKT2 exhibited increased invasiveness compared with cells transfected with vector alone, whereas expression of dominant negative AKT2 inhibited invasiveness. These data suggest that HGF/Met signaling contributes to the invasiveness of lung cancer cells via activation of the AKT pathway.

Discussion

We evaluated NSCLCs for the presence of active AKT. Our data indicate that AKT activation occurs in a subset of NSCLCs irrespective of tumor stage. In addition, p-AKT was expressed in metaplastic and dysplastic bronchial epithelial lesions. Progression from a preinvasive to a malignant state is a crucial step in tumorigenesis and recent investigations suggest that dysregulated cell motility, not proliferation, is critical for this transition (22). Earlier, we and others have shown that an important consequence of AKT activation is the acquisition of an invasive phenotype (23,24). Other investigators have reported that activation of PI3K signaling is implicated in HGF-dependent invasiveness and inactivation of PI3K results in reduced invasiveness of transformed human intestinal and kidney epithelial cells (25). Moreover, a recent study demonstrated that overexpression of AKT2 in breast cancer cells leads to increased invasiveness both *in vitro* and *in vivo* (26). We therefore hypothesized that increased AKT activation plays a role in the invasiveness of NSCLC cells.

The role of HGF and Met in cell invasion is well known (20). Specifically, following HGF stimulation or upon transfection with constitutively active Met, neoplastic cells disaggregate from the tumor mass, erode basement membranes, infiltrate stromal matrices and eventually colonize new territories (20). HGF–Met interaction is thought to play a role in tumor cell invasion and our data suggest that HGF/Met signaling contributes to the invasiveness of lung cancer cells via activation of the AKT pathway.

The current unsatisfactory cure rates in NSCLC underscore the need to develop better methods for early detection, when the disease is in a potentially curable, preinvasive stage. By identifying molecular biomarkers in preinvasive lung lesions, it may be possible to change the therapeutic paradigm from targeting advanced NSCLC toward asymptomatic, precursor lesions (9). Our preliminary studies of LIFE specimens suggest that AKT activation is an early and potentially critical event in lung cancer progression. Consistent with our findings, Chun *et al*. demonstrated that AKT is constitutively active in cell lines derived from premalignant and malignant human bronchial epithelial cells but not in normal bronchial cells (27). Other *in vitro* studies have revealed that AKT is activated in human airway epithelial cells upon exposure to nicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (28). Moreover, a recent report revealed increased p-AKT expression in bronchial hyperplasia, squamous metaplasia and bronchial dysplasia (29), which further supports our contention that AKT is an early event in lung cancer progression. Furthermore, alterations in AKT expression have been reported in early colon carcinogenesis (30). Collectively, these data highlight AKT as an important target for lung cancer prevention and treatment.

There was no obvious association between p-AKT expression and tumor stage in our series of NSCLCs. However, our *in vitro* findings suggest that increased AKT2 activity could play a role in local invasion, as might occur in the early stage of tumorigenesis. We speculate that in preinvasive bronchial epithelial lesions with AKT activation the presence of HGF in the microenvironment can promote invasiveness. HGF is expressed in normal lung at very low levels, but these levels increase in response to lung or distant injury (31). In lung, HGF has been shown to localize along the basement membranes of bronchial epithelium (32) and Met immunoreactivity has been observed on the apical membrane of normal bronchial epithelium (31,33). In addition, in studies employing an *in vivo* model, HGF was found to promote submucosal invasion and growth of human lung tumor cells within airway xenografts (34).

In conclusion, our data demonstrate frequent activation of the AKT pathway in primary NSCLC. Moreover, AKT activation can occur in preinvasive lung lesions. Based on its role in invasiveness, AKT activation may play a potentially important role in the conversion of a precursor lung lesion to a malignant carcinoma. Should AKT be validated as a biomarker in NSCLC, its properties as a critical signaling molecule with a central role in tumorigenesis (5) provide a compelling rationale for targeting AKT by a variety of chemopreventive measures.

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