Phospho-Akt Overexpression in Non–Small Cell Lung Cancer Confers Significant Stage-Independent Survival Disadvantage

Odile David,1 James Jett,4 Helena LeBeau,1 Grace Dy,4 Janet Hughes,2 Mitchell Friedman,3† and Arnold R. Brody1

Departments of 1Pathology, 2Biotostatics, and 3Medicine, Program in Lung Biology, Tulane University Health Sciences Center, New Orleans, Louisiana; and 4Division of Pulmonary Medicine, Mayo Clinic, Rochester, Minnesota

ABSTRACT

Purpose: Akt is a signal transduction protein that plays a central role in inhibiting apoptosis in a variety of cell types including human cancer cells. In cell lines derived from human non–small-cell lung cancers (NSCLCs), Akt has been shown to confer chemoresistance by inhibition of apoptosis in response to different chemotherapeutic agents including platinum-based agents, which are often the first-line therapy for NSCLCs. Only 20% to 30% of patients with NSCLC treated with chemotherapy have clinical evidence of response. The purpose of this study is to determine whether or not overexpression of activated Akt [i.e., phosphorylated Akt (pAkt)] is correlated with survival.

Experimental Design: We studied tumors from 61 patients with NSCLC in three tissue microarrays. All patients were followed for a period of 10 years or until death. The arrays were studied immunohistochemically with antibodies against pAkt, p53, and Ki-67.

Results: There was a statistically significant difference in survival between the 14 patients with strong pAkt staining and the 47 patients with weak to absent pAkt staining both by log-rank (P = 0.0416) and Breslow analysis (P = 0.0446). Difference in survival time with respect to pAkt status was also statistically significant even after accounting for stage at diagnosis (P = 0.004). Neither p53 nor Ki-67 was a statistically significant prognostic factor.

Conclusions: Overexpression of pAkt is an independent prognostic factor. Additional studies of human NSCLCs are warranted to drive the development of targeted tumor-specific antineoplastic therapies.

INTRODUCTION

Activation of the intracellular prosurvival signal transduction protein Akt, also known as protein kinase B (PKB), has been proposed as a central signaling event in carcinogenesis (1) and has been shown in experimental models to confer chemoresistance (2). There is strong interest in impacting the steady mortality of lung cancer, and we have postulated here that overexpression of the activated form of Akt [i.e., phosphorylated Akt (pAkt)], as detected by immunohistochemical techniques in histologic specimens from patients with non–small-cell lung cancer (NSCLC), can be used to predict reduced survival time of individual patients.

Lung cancer is the most lethal form of cancer for both men and women in the United States, with an estimated 172,000 new cases expected in 2003 and >157,000 estimated deaths, accounting for 28% of all cancer deaths (3). Lung cancer mortality has consistently been higher than the combined mortality of breast, colon, and prostate cancers. NSCLC comprises over 75% of lung cancers diagnosed and has an average overall 5-year survival of 14%. Survival rate is 49% for cases detected when the disease is still localized; however, only 15% of cases are detected early enough to impact this rate (4). Although early detection of NSCLC would provide the best chance of cure, the majority of NSCLCs are often diagnosed at later stages due to the lack of an effective noninvasive early detection method (e.g., cytologic evaluation of sputum). Although radiologic early detection systems are currently being evaluated, most prominently in the ongoing National Lung Cancer Screening Trial (5), methods for early detection of lung cancer by noninvasive molecular-based methods have not proven adequately sensitive, as demonstrated by recent studies evaluating a number of molecular markers including p53, k-ras, preproGRP, and monoclonal antibody 624H12 in sputum samples (6–11).

The current promise of targeted therapies, best exemplified by the success of Gleevec in combating chronic myelogenous leukemia, stems from an understanding of signal transduction pathways in carcinogenesis (12, 13). Breast, prostate, and ovarian cancers are subjects of intense study with respect to protein–protein and protein–cell interactions (14–16). Lung cancer is a relatively understudied malignancy in this regard (1). In the studies reported here, our data suggest that expression of the activated form of the cell survival protein Akt in primary NSCLCs is an independent indicator of poor prognosis.

MATERIALS AND METHODS

Collection of Clinical Data. At the Mayo Clinic, clinical data were compiled for 107 patients diagnosed with NSCLC in 1991 and 1992. Variables included age, sex, date of diagnosis, stage at diagnosis, time of follow-up, and vital status at time of...
last follow-up. Survival curves based on stage at time of diagnosis showed the expected patterns. There were no unusual characteristics in this population. Clinical data were not made available to collaborators at Tulane University, where laboratory analysis was performed, until all laboratory data were evaluated. Institutional review board approval for this study was obtained at both institutions.

**Tissue Arrays.** At the Mayo Clinic Core Laboratory, tissue arrays were prepared using a Beecher manual arrayer. Of the 107 patients, 64 had histologic tissue samples representing non–small-cell carcinoma obtained at the time of diagnosis. A total of three paraffin blocks were prepared from these samples. In each block, the central row and central column consisted of normal human hepatic tissue included as internal negative controls. From each block, one hematoxylin and eosin (H&E)-stained section and six unstained sections were cut at 5-μm intervals, and the resultant 21 total slides were sent to a team of collaborators led by O. D. at Tulane University.

The value of tissue arrays as an investigative tool is becoming widely recognized (17, 18). One H&E-stained section and six unstained sections were received from each of three array blocks. A section from array 1 stained with routine H&E stain is shown in Fig. 1 and is representative of the other two arrays.

**Tissue Array Analysis.** H&E-stained sections were examined to confirm the presence or absence of non–small-cell carcinoma on the slides. Of the 107 patients for whom follow-up data were available, 65 were represented in the tissue array. Of the 65 patients represented, 4 showed only benign tissue. Therefore NSCLC tissues from 61 patients were represented in the arrays. In 48 cases, three spots of tissue per patient sample were represented in the array. In six cases, two spots were represented on the array. In three cases, five spots were represented. In another three cases, six spots were represented, and in one case, nine spots were represented. There were 28 adenocarcinomas, 18 squamous cell carcinomas, and 15 poorly differentiated carcinomas that could not be subtyped. We recognize that NSCLCs are commonly heterogeneous histologically and that subtyping of small samples of tumor may not provide a definitive diagnosis. No metastatic tumors were included in the arrays.

**Immunohistochemistry.** Each primary antibody was applied in a separate run (i.e., all pAkt staining was done in one run, all p53 staining was done in a second run, and all Ki-67 staining was done in a third run). For pAkt, the primary antibody (Cell Signaling Technology, Beverly, MA) was produced by immunizing rabbits with a synthetic phospho-Ser473 peptide corresponding to residues around Ser473 of mouse Akt. Nuclear staining and cytoplasmic staining are expected. The p53 primary antibody (DAKO, Carpinteria, CA) was a D07 clone. The Ki-67 primary antibody was also obtained from DAKO. Positive and negative control slides were included in every run. In the pAkt run, the positive control was a section from a block of infiltrating breast carcinoma. In the p53 and Ki-67 runs, the positive controls were sections from a block of infiltrating colon carcinoma. Negative controls consisted of one row and one column per array slide of spots of normal liver tissue as well as array slides to which only buffered saline but no primary antibody was applied. Immediately before each run, antigen retrieval was performed by cooking the deparaffinized slides in a commercial rice cooker in preheated 95°C DAKO target retrieval solution. After cooling and washing, slides were loaded onto a DAKO-Autostainer instrument. The slides were treated with 3% H2O2 endogenous block for 5 minutes followed by serum-free DAKO protein block for 5 minutes. Primary antibodies were applied at dilutions of 1:50 for the pAkt antibody and 1:40 for the p53 and Ki-67 antibodies. Incubation time was 120 minutes for pAkt and 60 minutes for p53 and Ki-67. Secondary antibody (DAKO LSAB+ Link system) was applied for 30 minutes. Tertiary antibody (DAKO LSAB+ streptavidin horseradish peroxidase) was applied for 30 minutes. DAKO 3,3’-diaminobenzidine chromogen substrate was applied until light brown staining was seen (approximately 30 seconds).

**Immunohistochemical Scoring.** Interpretation of the results for pAkt, p53, and Ki-67 staining was performed as follows. Blinded to the clinical data, a pathologist (O. D.) examined all of the spots in the three arrays. Phospho-Akt was given as levels on an intensity scale ranging from 0 to 3 as illustrated (Fig. 2). Scoring was performed as follows: 0, no appreciable staining in tumor cells; 1, barely detectable staining in cytoplasm and/or nucleus as compared with stromal elements; 2, readily appreciable brown staining distinctly marking tumor cell cytoplasm and/or nucleus; and 3, dark brown staining in tumor cells completely obscuring cytoplasm and/or nucleus. This scoring system has been reported previously (19). For purposes of statistical analysis, all cases staining at level 0 or 1 were grouped as Akt1 and all cases staining at level 2 or 3 were grouped as Akt2. Because more than one spot was represented for most patients, a decision was made that if at least one spot scored as level 2 or 3, the patient would be included in Akt2 as reported previously by Tsao et al. (20). P53 and Ki-67 were given as an average percentage of tumor nuclei staining positively in each spot per patient represented (Fig. 3).

**Statistical Analysis.** Kaplan-Meier survival curves were calculated for the patient subgroups of interest and compared statistically, censoring for age and stage, using the log-rank test. All statistical analyses were conducted using SPSS for Window (version 10).
Fig. 2  A, level 0 staining for pAkt. B, level 1 staining for pAkt. Adenocarcinoma, left panel; squamous cell carcinoma, right panel. C, level 2 staining for pAkt. Poorly differentiated tumor, left panel; predominantly nuclear staining, right panel. Predominance of nuclear staining was seen only in this patient, who was the only patient in group 2 (defined as staining at level 2 or 3 in at least one spot) to survive. P53 expression for this patient was 95%. Ki-67 expression was 35%. D, level 3 staining for pAkt. Squamous cell carcinoma, left panel; adenocarcinoma, right panel. Original magnification, ×400 for all panels.
RESULTS

It has been shown previously that pAkt is commonly over-expressed in human NSCLCs (2) and that archival formalin-fixed NSCLCs are amenable to immunohistochemical staining with antibodies against pAkt (21). This collaborative retrospective outcome study permitted us to link pAkt expression with prognosis. We hypothesized that overexpression of pAkt would be predictive of shortened survival. In this study, antibodies against pAkt, p53, and Ki-67 were applied to the tissue arrays of human archival paraffin-embedded resected NSCLCs.

Each spot on the array was graded individually. Patients whose tumors stained at level 2 or greater in at least one spot were designated pAkt group 2. Patients whose tumors stained at level 1 or 0 in all spots represented were designated pAkt group 1. Staining level was generally consistent within a spot. Cytoplasmic and nuclear staining were both noted. There was no detectable expression of pAkt either in normal lung tissue or stroma adjacent to tumors within spots or in spots of normal liver tissue included in the arrays (data not shown).

Fourteen of the 61 cases in which NSCLC tissue was represented in the arrays demonstrated strong staining with pAkt antibody. Thirteen of the 14 patients in pAkt group 2 were deceased at the end of the follow-up period. The mean survival time from diagnosis for the patients in this group was 36.2 months. The single patient still alive was followed for 111 months after diagnosis. This patient’s tumor demonstrated only focal but strong nuclear staining and weak cytoplasmic staining with pAkt, the significance of which is unclear. All other cases showing strong staining in at least one spot manifested predominantly cytoplasmic staining. Also of interest was the finding that 7 of the 14 tumors staining strongly with pAkt in at last one spot on the array also demonstrated diffuse strong staining with p53 (95–100% of nuclei in each of those spots). One of the 14 tumors demonstrated an average of 80% nuclear staining of tumor cells with p53 in all spots represented. Six of the 14 tumors demonstrated <10% nuclear staining of tumor cells with p53 in all spots represented.

Forty-seven of the 61 cases in which NSCLC tissue was represented in the arrays demonstrated weak staining with pAkt antibody. Thirty-five of the 47 patients in pAkt group 1 were deceased at the end of the follow-up period. The mean survival time from diagnosis for the patients in this group was 57.0 months.

All of the patients in this population demonstrated patterns of survival rates within stage groups that were consistent with national trends. There were no unusual characteristics. The pattern of survival curves showed the expected trend of decreasing survival with increasing stage at diagnosis, which was statistically significant using a log-rank test for trend ($P = 0.03$; Fig. 4).

Difference in survival for 14 patients with strong pAkt staining (group 2 as defined above) and weak or no pAkt staining (group 1 as defined above) was statistically significant by both log-rank ($P = 0.0416$) and Breslow analysis ($P = 0.0446$; Fig. 5). Median survival for pAkt group 2 was 19 months compared with 56 months for pAkt group 1.

Within each stage (except stage 4, where all six patients were in pAkt group 1), a higher percentage of patients in pAkt group 2 died compared with patients in pAkt group 1. For example, of those in stage I, 88.9% of pAkt group 2 patients...
died, compared with 61.5% of pAkt group 1 patients. Survival analysis found that pAkt was a significant factor after allowing for stage and age at diagnosis. Age at diagnosis was not at all significant in this population. Stage, however, was significant; even after accounting for stage, pAkt was a significant factor after allowing for stage and age at diagnosis. The pAkt status was even more significant (P = 0.004) than that of deceased patients by p53 and Ki-67 groupings were also not statistically significant (P = 0.92). The percentages of deceased patients by p53 and Ki-67 groupings were also not statistically significant (P = 0.202). Analysis of p53 and Ki-67 status stratified by pAkt status could not be performed due to the small numbers of patients in each subgroup. p53 and Ki-67 were not independent prognostic factors by either a simple analysis of the percentage dying or survival analysis. We conclude that clear expression of pAkt correlates significantly with reduced patient survival.

It is well accepted that archival tissue presents special challenges when applying immunohistochemical techniques (17, 26–31). Duration of fixation, nature of fixative, processing conditions including adequacy of tissue dehydration, and paraffin block storage conditions are all factors that affect antigen preservation. The conditions before, during, and after fixation are unknown to us for the tissues represented in the arrays. When considering antigens that are phosphorylated, it becomes even more important to know how quickly the tissue was placed in fixative after being removed from the patient because dephosphorylation may occur ex vivo. Despite these issues, staining of the array slides with pAkt, p53, and ki-67 was successful. Although the pAkt antibody was developed relatively recently for immunohistochemical applications, it is becoming increasingly routine in such use in research laboratories around the world (16, 20, 32–34). Staining was without background and appeared to be specific.

Akt, also known as PKB and rac (related to protein kinase a and c), is a cytosolic signal transduction protein that plays an important role in the cell survival pathway (15). It is recruited to the plasma membrane by phosphatidylinositol 3-kinase (PI3K), where it is phosphorylated by PDK1 and a second, as yet unidentified kinase. More specifically, Akt contains an NH2-terminal pleckstrin homology domain that binds phosphorylated lipids at the membrane in response to activation of PI3Ks. Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 and also by phosphorylation within the COOH terminus at Ser473 (35). Phosphorylation at the Thr308 site appears to be necessary and sufficient. Activated Akt may be found in the cell nucleus, although its exact function there is unknown. Activated Akt promotes cell survival by inhibiting apoptosis by means of phosphorylating and thereby inactivating targets that include members of the bel-2 family, caspase 9, and forkhead transcription factors, among others (36). Activated Akt has been shown to negatively regulate the Ras-Raf-mitogen-activated protein kinase pathway via phosphorylation and inactivation of Raf at Ser329 (37, 38). This is of particular significance in lung cancer due to the relatively high mutation rates of k-ras in NSCLC (39–43). Of even greater relevance are the findings of Okudela et al. (44) demonstrating that k-ras gene mutation enhances motility of immortalized lung adenocarcinoma cells via Akt activation. It has also been shown
previously that in NSCLC cell lines, phosphorylation of Akt by PI3K promotes cell survival, and inhibition of PI3K permits apoptosis (45). This has also been demonstrated in murine mammary tissue (15) and human colorectal carcinomas (46). In addition, the influence of Akt on the p53-mediated apoptotic pathway has been demonstrated (47), as has the effect of p53 on Akt (48). More specifically, Akt is believed to up-regulate expression of mdm2, thereby promoting ubiquitination of p53 and decreased apoptosis, whereas p53 has been shown to inhibit α5β1 integrin survival signaling by promoting the caspase 3-dependent cleavage of Akt.

Akt has been found to play a role in the survival of cancer cells in breast, prostate, ovary, and brain tissue (14, 15, 49, 50). It has also been shown to confer chemoresistance in NSCLC cell lines treated with topotecan (51) and etoposide, cisplatin, paclitaxel, and gemcitabine and trastuzumab (Herceptin) (2) as well as breast cancer cell lines treated with trastuzumab (52). In a series of investigations conducted by Brognard et al. (2) at the National Cancer Institute, constitutive Akt/PKB activity was demonstrated in 16 of 17 NSCLC cell lines by maintenance of Ser473 phosphorylation with serum deprivation. Akt activation was PI3K dependent and promoted survival; the PI3K inhibitors LY294002 and wortmannin inhibited Akt phosphorylation and increased apoptosis in cells with constitutively active Akt, but not in cells without constitutively active Akt. To test whether Akt activity promoted therapeutic resistance, the investigators added LY294002 with individual chemotherapeutic agents or irradiation. LY294002 greatly potentiated chemotherapy-induced apoptosis in cells with high constitutive Akt levels but did not significantly increase chemotherapy-induced apoptosis in cells with low constitutive Akt levels. Combined with radiation in cells with active Akt, LY294002 additively increased apoptosis and inhibited clonogenic growth. These results were reproducible in transiently transfected Akt mutants. Transfecting dominant negative Akt decreased Akt activity and increased basal apoptosis as well as chemotherapeutic- and irradiation-induced apoptosis only in cells with high Akt/PKB activity. Conversely, transfecting constitutively active Akt into cells with low Akt/PKB activity increased Akt activity and attenuated chemotherapy- and radiation-induced apoptosis. By these methods, Akt was identified as a constitutively active kinase that promotes survival of NSCLC cells. It was concluded that modulation of Akt activity by pharmacological or genetic approaches alters the cellular responsiveness to therapeutic modalities typically used to treat patients with NSCLC (2). Although there are three known isoforms of Akt, referred to as 1, 2, and 3, the functional differences have not yet been elucidated (53).

Our results are timely in light of the emerging significance of Akt activation as a premalignant event in human bronchial epithelial cells exposed to cigarette smoke toxins including nicotine and 4-(methylaminos)-1,3-(3-pyridy)-1-butanoic (34). A recent clinical study of pAkt expression in tissue from patients with bronchial epithelial dysplasia and malignancy corroborates this hypothesis. Tsao et al. (20) observed strong pAkt staining in 12 of 44 normal bronchial biopsy specimens, 4 of 9 reactive specimens, 22 of 25 dysplastic specimens, and 25 of 76 non–small cell carcinoma specimens. They suggest that Akt activation may be more strongly associated with the early stages of malignant transformation than with progression to frank neoplasia. This is consistent with the findings in our study that Akt activation was of greater clinical significance in terms of survival in patients diagnosed at early stages.

Because non–small cell carcinoma is diagnosed in overwhelming proportions in patients with a history of smoking and smoking cessation efforts have met with limited success, the need for advances in treatment is great to impact the persistently high mortality rates (54). Promising organic compounds such as deguelin have been shown in recent studies to block both PI3K-dependent and -independent activation of Akt in NSCLC cell lines, thereby increasing apoptotic activity (55). Our studies presented here, considered with current findings on the role of Akt in chemoresistance (25), suggest that prospective studies to establish the prognostic value of pAkt expression are warranted.

REFERENCES

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