Phosphorylated Akt expression is a prognostic marker in early-stage non-small cell lung cancer

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ABSTRACT

Aims To determine the prognostic significance of pAkt expression in order to identify high-risk stage IB patients with non-small cell lung cancer (NSCLC) in an exploratory study.

Methods We identified 471 consecutive patients with stage IB primary NSCLC according to the American Joint Commission on Cancer 6th edition tumour-node-metastasis (TNM) staging system, who underwent surgical resection between 1990 and 2008. Patients who received neoadjuvant or adjuvant treatments were excluded. Pathology reports were reviewed, and pathological characteristics were extracted. Expression of phosphorylated Akt (pAkt) in both cytoplasmic and nuclear locations was assessed by immunohistochemistry, and clinicopathological factors were analysed against 10-year overall survival using Kaplan–Meier and Cox proportional hazards model.

Results 455 (96.6%) cancers were adequate for pAkt immunohistochemical analysis. The prevalence of pAkt expression in the cytoplasm and nucleus of the cancers was 60.7% and 43.7%, respectively. Patients whose cancers expressed higher levels of cytoplasmic pAkt had a trend towards longer overall survival than those with lower levels (p=0.06). Conversely, patients whose cancers expressed higher levels of nuclear pAkt had a poorer prognosis than those with lower levels of expression (p=0.02). Combined low cytoplasmic/high nuclear expression of pAkt was an independent predictor of overall survival (HR=2.86 (95% CI 1.35 to 6.04); p=0.006) when modelled with age (HR=1.05 (95% CI 1.03 to 1.07); p<0.001), extent of operation (HR=2.11 (95% CI 1.48 to 3.01); p<0.001), visceral pleural invasion (HR=1.63 (95% CI 1.24 to 2.15); p<0.001), gender, tumour size, histopathological type and grade (p>0.05).

Conclusions Level of expression of pAkt in the cytoplasm and nucleus is an independent prognostic factor that may help to select patients with high-risk disease.

INTRODUCTION

Lung cancer is the most common and leading cause of cancer death worldwide, with an incidence of 1.6 million new cases annually and a mortality of 1.38 million in 2008.[1] In early-stage non-small cell lung cancer (NSCLC), surgical resection remains the treatment of choice with adjuvant chemotherapy having a modest absolute survival benefit of 5% at 5 years in stage II–IIIA disease, while subgroup analyses were not significant in stage IB disease.[2] Nevertheless, 45% of patients with resected stage IB disease will die of their cancer. Therefore, there is an urgent need to identify novel prognostic markers that can select patients with early-stage disease who are at high risk of recurrence. Numerous molecular prognostic markers have been examined in NSCLC,[3–11] but to date none have moved into routine clinical practice.

Our aim was to identify a molecular prognostic marker for stage IB resected NSCLC; therefore, we undertook a literature search to establish which would be the most appropriate marker to assess in this study. Our criteria were (1) that there be more than one study of a molecular marker (ie, an independent validation study), (2) the marker had to be an independent predictor of outcome in multivariable analysis and (3) had to be implicated in the biology of lung cancer. It was preferable for the molecular marker to be assessable by immunohistochemistry so that it would be appropriate for routine hospital pathology implementation. Phosphorylated Akt (pAkt) not only fulfilled all our predetermined criteria but it is also a key mediator in numerous signalling pathways that are central to the balance of cell survival, proliferation and apoptosis. Furthermore, it is particularly important in the PI3K/Akt/mTOR signalling pathway, where major therapeutics are either already in clinical practice, such as epidermal growth factor tyrosine kinase inhibitors (gefitinib and erlotinib) and mTOR inhibitors (everolimus and temsirolimus), or in development, such as dual PI3K/mTOR, PI3K and Akt inhibitors.[12]

There have been a number of studies that have demonstrated an association between overexpression of pAkt in NSCLC and a poorer prognosis.[13–18] However, these studies included patients with heterogeneous stages of disease, different immunohistochemical protocols and pAkt antibodies against different phosphorylation sites, while others had small sample sizes, which were underpowered to demonstrate prognostic significance.[19–21]

Given the potential role of pAkt as a prognostic marker, we aimed to examine the association between pAkt expression and outcome in a cohort of patients, who had resected early-stage node-negative NSCLC, in order to identify patients who are at high risk of recurrence and may potentially benefit from adjuvant chemotherapy.

PATIENTS AND METHODS

We retrospectively reviewed 527 consecutive patients with stage IB primary NSCLC according to the American Joint Commission on Cancer 6th edition tumour, node, metastasis staging system, who underwent surgical resection between January 1990 and May 2008 at the Sydney Cancer Centre (Royal Prince Alfred Hospital/Concord

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Repatriation General Hospital). We excluded 56 patients who had segmental or wedge resections or who had received neoadjuvant or adjuvant treatments to keep the cohort homogenous. Pathology reports were reviewed, and pathological characteristics (tumour size, histopathological type, grade, visceral pleural, vessel and perineural invasion) were extracted. Two representative formalin-fixed paraffin-embedded tumour blocks from each case were retrieved from the pathology archives. They were sectioned and stained with H&E. An experienced pulmonary pathologist (WAC) reviewed the slides and marked representative areas. Histopathological type was classified according to the World Health Organisation Classification of Tumours 2004. Tissue microarrays (TMAs) were constructed using four 1 mm tumour cores taken from two separate tumour blocks in areas carefully selected by an experienced pulmonary pathologist (WAC) to account for morphological heterogeneity and potential loss of tumour cores during processing.[5] Matched non-neoplastic lung tissue was also used from each case. Previous studies by Cooper *et al*[5 7] and recent studies by Karlsson *et al*[22] and Schmidt *et al*[23] have shown that 3–4 tumour cores are adequate for TMA studies in lung cancer. Immunostaining on whole sections was not performed because the use of TMAs in NSCLC has been validated in previous pAkt immunohistochemistry studies.[13–17 19–21] This study was approved by the Human Research Ethics Committee of Royal Prince Alfred (X10-0278; HREC/10/RPAH/491) and Concord Repatriation General Hospitals (CH62/6/2004-116).

Immunohistochemistry

Four-micrometre-thick sections were cut from the TMA blocks, mounted onto charged slides and sections were baked in an oven at 60°C for 2 h. Sections were de-paraffinised and rehydrated through graded alcohols. Antigen retrieval was performed by placing slides in 10 mmol/L citric acid buffer, pH 6.0 Dako Target Retrieval Solution, S1699 (Dako, Glostrup, Denmark) in a pressure cooker for 4 min at 125°C, followed by running cold water around the antigen retrieval box containing the slides for 15 min to cool down. Slides were removed from the retrieval solution, rinsed in distilled water for 1 min and then loaded onto the Dako Autostainer (Dako, Glostrup, Denmark). Slides were treated with 3% hydrogen peroxides endogenous block for 5 min, followed by a buffer rinse (Dako Wash Buffer, S3006). They were then incubated with 5% goat serum in TBST for 10 min to block non-specific background staining. The primary antibody, phosphorylated Akt (Ser473) rabbit monoclonal antibody (Cell Signaling Technology, Beverly, Massachusetts, USA) at a 1:50 dilution, was applied to the sections and incubated for 90 min. Slides were rinsed with Dako buffer and incubated with the Mach3 Rabbit Probe, M3R531H (Biocare Medical, Concord, California, USA) for 20 min. After two further buffer rinses, slides were incubated with the Mach3 Rabbit Polymer-HRP, M3R531H (Biocare Medical) for 20 min. Dako liquid DAB+ substrate chromogen system, K3468, was applied to slides and incubated for 10 min for visualisation. Sections were removed from the Dako Autostainer and rinsed in distilled water. They were then counterstained in haematoxylin, dehydrated and cleared in xylene before coverslipping. Sections were analysed by standard light microscopy. Prostate LnCAP cells were used as a positive control, and Rabbit IgG (Cell Signaling Technology) was used as a negative control.

Assessment of pAkt

The extent and pattern of pAkt-specific immunostaining within a tissue section was determined by the percentage (0%–100%) of positively staining cells and intensity of staining (0, absent; 1+, weak; 2+, moderate; and 3+, intense) within the cytoplasm (C) and the nucleus (N). Sections were assessed independently by two investigators (WAC and PYY), and a modified H-score (the product of percentage of positively staining cells and intensity) was calculated for each core.[24] The final modified H-score of each case was the average from all available tumour cores. The concordance of pAkt score between the two investigators was high in both cytoplasmic (Spearman's correlation coefficient, $r=0.93$, $p<0.001$) and nuclear ($r=0.89$, $p<0.001$) compartments.

Table 1 Clinicopathological characteristics of 471 resected node-negative non-small cell lung cancer

Clinicopathological factors	Number (%)
Median age	69 (40–87)
Gender	
Male	337 (71.5)
Female	134 (28.5)
Extent of operation	
Lobectomy	401 (85.1)
Pneumonectomy	70 (14.9)
Tumour size (cm)	
≤5	324 (68.8)
5.1–7	106 (22.5)
>7	39 (8.3)
Not reported	2 (0.4)
Histopathology	
Squamous cell carcinoma	206 (43.7)
Adenocarcinoma	185 (39.3)
Large cell carcinoma	68 (14.4)
Mixed/others*	12 (2.5)
Histological grade	
Well differentiated	28 (5.9)
Moderately differentiated	197 (41.8)
Poorly differentiated	181 (38.4)
Undifferentiated	18 (3.8)
Not reported	47 (10)
Visceral pleural invasion	
Yes	168 (35.7)
No	258 (54.8)
Not reported	44 (9.3)
Missing	1 (0.2)
Vessel invasion	
Yes	47 (10)
No	144 (30.6)
Not reported	279 (59.2)
Missing	1 (0.2)
Perineural invasion	
Yes	16 (3.4)
No	128 (27.2)
Not reported	326 (69.2)
Missing	1 (0.2)

*Adenosquamous carcinoma or other histopathology.

Statistical analysis

To examine the relationship between the level of pAkt expression and the clinicopathological factors, Pearson's χ^2 test was performed. Kaplan–Meier and log-rank analyses were performed on the cytoplasmic and nuclear pAkt H-scores in a stepwise fashion (ie, using a cut-off of 10).[25] The cutpoints that resulted in the most significant relation with overall survival

were selected. The optimal modified H-score cutpoints for pAkt expression were 70 in the cytoplasmic (≤70, low vs >70, high) and 40 (≤40, low vs >40, high) in nuclear compartments, respectively. The cohort was grouped into four categories (high C/low N, low C/low N, high C/high N and low C/high N) according to the level of pAkt expression in these two subcellular compartments. We understand that this will overestimate the magnitude of the effect, but this raises hypotheses that warrant corroboration in an independent dataset before it can be considered for routine clinical practice. Although the median follow-up was 11.3 years, survival was censored at 10 years as lung cancer-related death was unlikely >10 years postresection. Overall 10-year survival was calculated from the time of resection until the time of last follow-up or death within 10 years from resection. Lung cancer-specific mortality could not be assessed due to inconsistency in the source documentation.

Survival analysis was performed using univariable and multivariable analyses in a Cox proportional hazards model for pAkt expression and known clinicopathological prognostic factors. Age, gender, extent of operation, tumour size, histopathology, histological grade and visceral pleural invasion were selected for inclusion in the multivariable model because they have previously proven prognostic in NSCLC, regardless of their statistical significance in this dataset. All p values were two-sided, and a p value <0.05 was considered significant. All statistical analyses were performed using PASW Statistics V.18.0 (SPSS Inc, Chicago, Illinois, USA).

RESULTS

The clinicopathological characteristics of 471 patients with resected node-negative NSCLC are described in table 1. The 5-year and 10-year survival rates were 56% and 38%, respectively. The majority of patients (85.1%) underwent lobectomy. Visceral pleural invasion was consistently reported, but >50% of cases had missing data for vessel and perineural invasion. Therefore, vessel and perineural invasion were not included in the subsequent analysis.

Out of the 471 patients, 455 (96.6%) cases with adequate samples were available for pAkt immunohistochemistry assessment, while 16 cases were excluded due to either inadequate tumour tissue or missing paraffin blocks from the archive. pAkt was expressed in both cytoplasmic and nuclear compartments of the cancers (figure 1). PAkt expression was absent in non-neoplastic peripheral lung parenchyma.

The cytoplasmic and nuclear expression patterns were analysed both separately and in combination. In the cohort, 60.7% (276/455) of cases expressed pAkt in the cytoplasm and 43.7% (199/455) in the nucleus. In 39.8% (181/455) of cases, pAkt was expressed in both cytoplasm and nucleus of the cancer (figure 2). Cancers that expressed high levels of pAkt in the cytoplasm were associated with poor differentiation (p=0.03). There were no significant relationships between other clinicopathological factors (gender, tumour size, histopathological type and visceral pleural invasion) and levels of expression of pAkt in the cytoplasm and/or nucleus of the cancers.

Patients whose cancers expressed high levels of pAkt in the cytoplasm (modified H-score >70) accounted for 23% (104/455) of the cohort and had a trend towards better survival over 10 years than those with low levels of pAkt expression (p=0.06, figure 3A). Conversely, patients whose cancers expressed high levels of pAkt in the nucleus (>40) accounted for 11% (50/455) of the cohort and had a poorer prognosis than those with low levels of nuclear pAkt expression (p=0.02, figure 3B). When cytoplasmic and nuclear pAkt expression was considered in combination, patients whose cancers expressed high levels of pAkt in the nucleus (>40) but low levels of pAkt in the cytoplasm (≤70) had a poorer survival over 10 years than

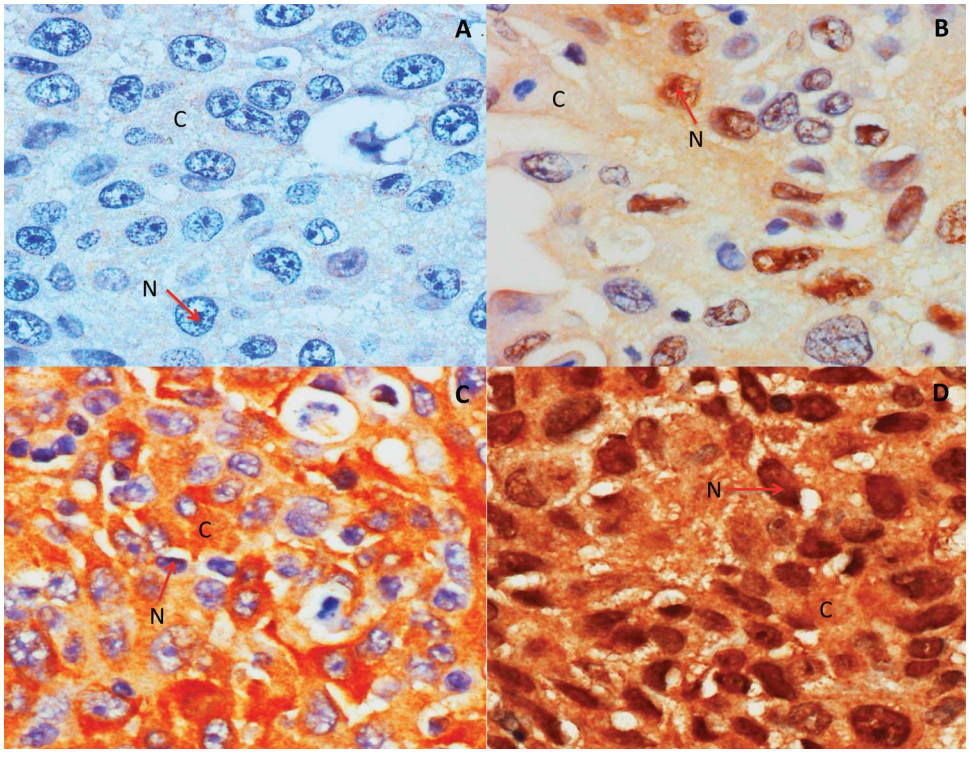

Figure 1 Immunohistochemistry staining of different levels of pAkt expression in cytoplasm (C) and nucleus (N) (A), low C/low N (B), low C/high N (C), high C/low N (D) and high C/high N.

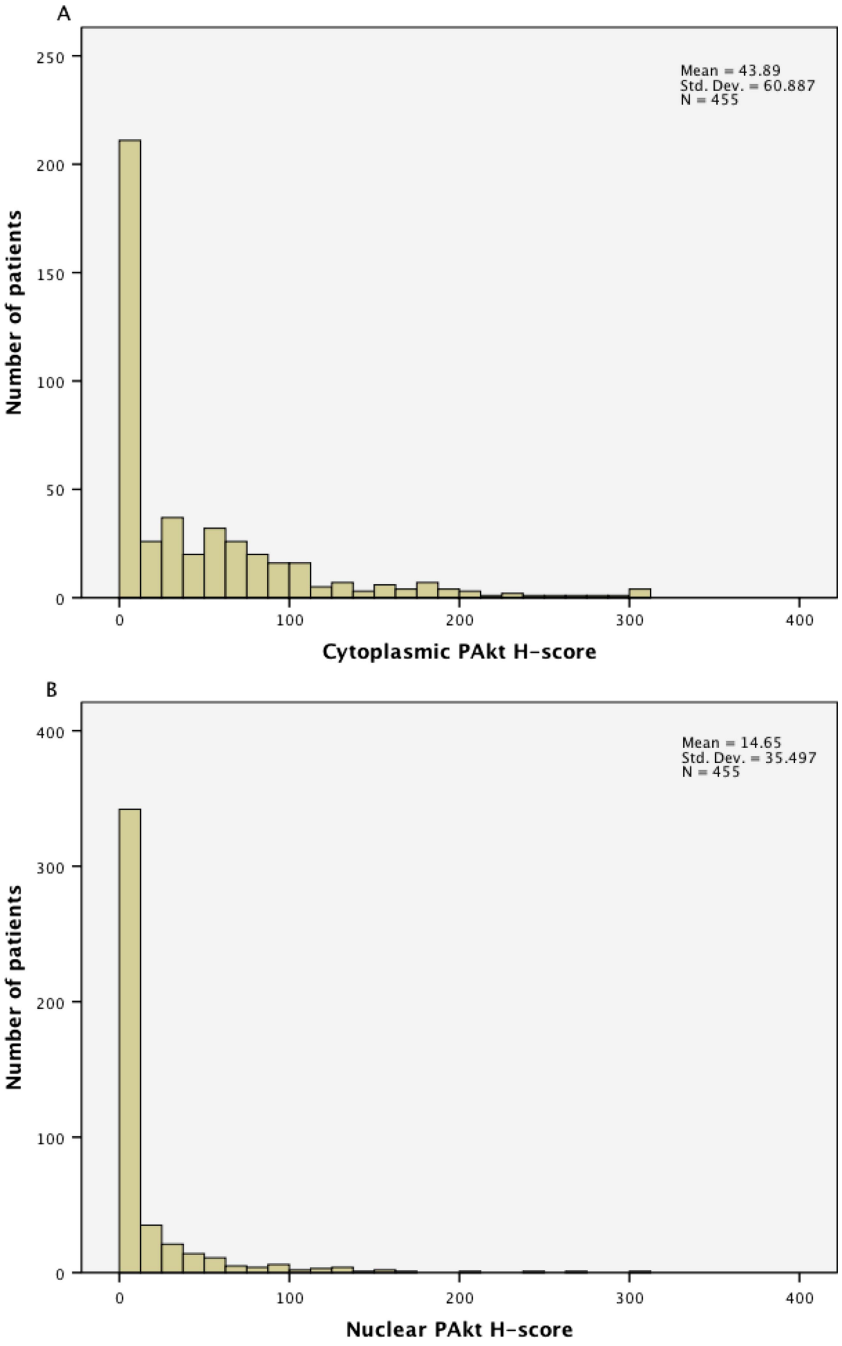

Figure 2 Histograms demonstrating the frequency of pAkt expression in (A) cytoplasm and (B) nucleus.

those with cancers that expressed low pAkt levels in the nucleus (≤40) but high levels in the cytoplasm (>70) (p=0.002, figure 3C). The representative examples of four different combinations of expression of pAkt within the cytoplasm and nucleus are illustrated in figure 1.

PAkt expression was assessed for potential relationships with other known clinicopathological prognostic factors. Univariable analysis demonstrated that age (p<0.001), gender (p=0.002), type of operations (p=0.002), histological grade (p<0.05) and combined cytoplasmic and nuclear pAkt expression (p<0.05) were all significant predictors of survival over 10 years (table 2). There was a trend towards worse survival over 10 years with visceral pleural invasion (HR=1.27), even though it was not statistically significant (p=0.07). With respect to the individual cellular compartments, patients whose cancers expressed high levels of pAkt in the cytoplasm had a trend towards reduced risk of death over 10 years (HR=0.74, p=0.06), whereas those whose cancers expressed high levels of pAkt in the nucleus had a significantly increased risk of death over 10 years (HR=1.54, p=0.02). Cytoplasmic and nuclear pAkt expressions were both independent predictors of survival over 10 years (p=0.01 and p=0.002) in multivariable analysis when modelled with age (p<0.001), type of operations (p<0.001), visceral pleural invasion (p<0.001), gender, size, histopathological type and grade (p>0.05) (data not shown).

In addition, the combination of low cytoplasmic (≤70)/high nuclear (>40) pAkt expression in cancers was an independent predictor of poorer overall survival over 10 years (p=0.006) when modelled with age (p<0.001), type of operations (p<0.001), visceral pleural invasion (p<0.001), size (p>0.05), histopathological type (p>0.05), grade (p>0.05) and gender (p=0.07) (table 2).

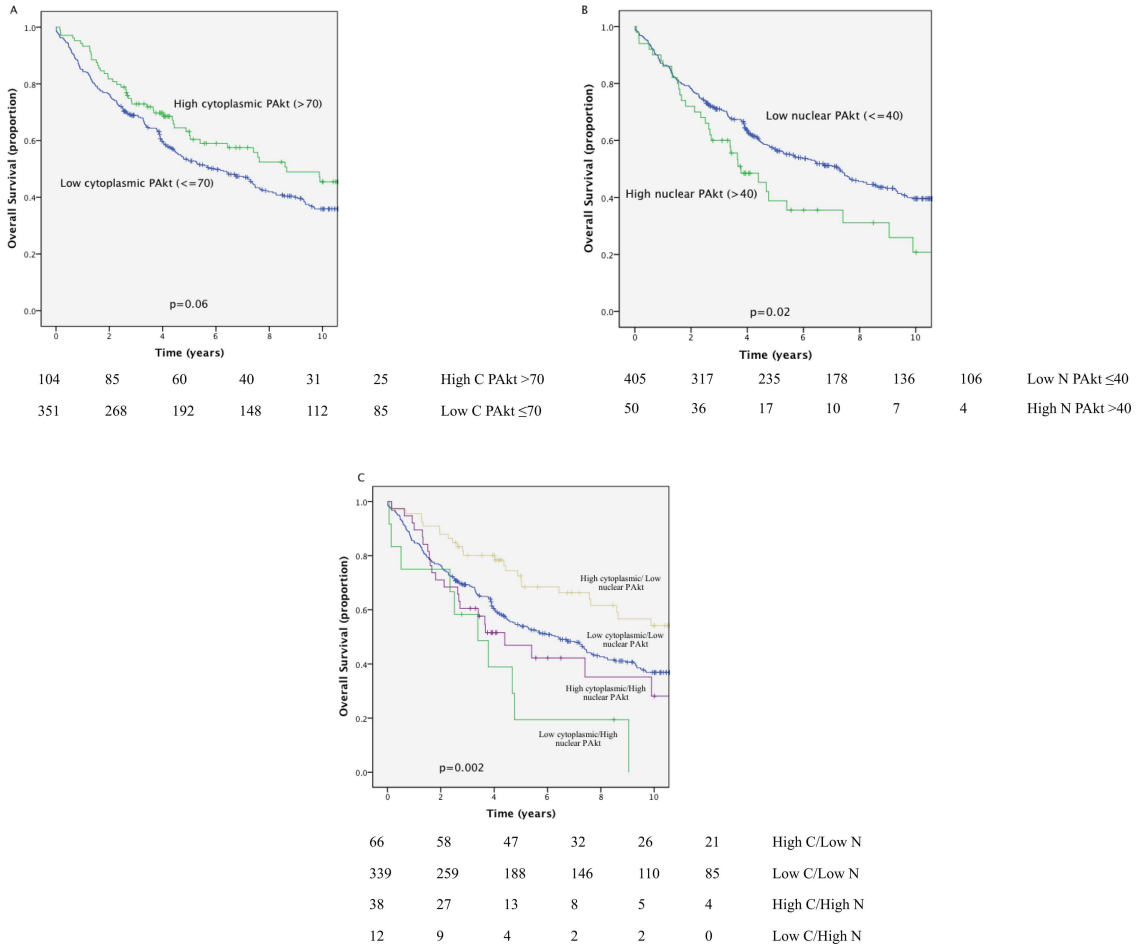

Figure 3 Kaplan–Meier curves demonstrating the association between overall survival censored at 10 years and the levels of expression of pAkt by immunohistochemistry (A), cytoplasmic compartment (B), nuclear compartment (C) and cytoplasmic/nuclear compartments.

DISCUSSION

This study demonstrates that pAkt is expressed in both the cytoplasmic and nuclear compartments of resected early-node-negative, treatment-naïve NSCLC. Patients whose cancers expressed high levels of pAkt in the cytoplasm had a trend towards a better prognosis, whereas patients whose cancers expressed high levels of pAkt in the nucleus had a significantly poorer prognosis. When cytoplasmic and nuclear pAkt expression was considered in combination, patients whose cancers expressed high levels of nuclear pAkt but low levels of cytoplasmic pAkt had a significantly shorter overall survival than those with cancers that expressed low pAkt levels in the nucleus but high levels in the cytoplasm. Furthermore, the levels of cytoplasmic and nuclear pAkt expression in combination were an independent predictor of survival.

Although five randomised controlled trials and a meta-analysis had demonstrated that adjuvant chemotherapy improved survival in patients with stage II–IIIA NSCLC, subgroup analysis showed no significant improvement in patients with stage IB disease. Therefore, adjuvant chemotherapy has not been recommended in stage IB NSCLC.[2] Furthermore, the 5-year survival rate of stage IB NSCLC remains poor at 55%. Unfortunately, the two randomised controlled trials that included only patients with stage I NSCLC were either underpowered or used a non-platinum-based oral therapy, which was not available outside Asia.[26 27] Therefore, identification of a biomarker that can differentiate high-risk patients who may benefit from adjuvant chemotherapy raises the potential for better stratification of early NSCLC for treatment.

There have been 14 studies investigating the role of pAkt in NSCLC published since 2002. Earlier studies[19 20 28] found that expression of pAkt was not associated with prognosis, but later studies[13–18] showed that overexpression of phosphorylated Akt was associated with a poorer prognosis in NSCLC. However, these studies included patients with a wide range of disease stages, I–IV, who had been treated in a heterogeneous fashion and whose cancers had been evaluated with a variety of immunohistochemical protocols and pAkt antibodies specific to different phosphorylation sites (Thr308 and Ser473). Moreover, many studies were underpowered for survival analysis. On the other hand, our study was homogeneous in stage (node-negative stage IB) and treatment (surgical resection only) representing the largest cohort of early-stage NSCLC investigated for pAkt expression to date. Even though there are three studies suggesting that NSCLC expressing pAkt at the threonine (Thr308) phosphorylation site is associated with a poorer outcome,[16–18] we chose to examine the pAkt (Ser473) since it has been examined in the majority of previous studies and has been consistently identified as a negative prognostic factor. Similar to previous retrospective studies using immunohistochemical analysis, our study is limited by its design and retrospective data collection. Therefore, even though tumour size is an established prognostic factor, unexpectedly, this was not demonstrated in our study.

Akt (v-Akt murine thymoma viral oncogene)/PKB (protein kinase-B) is a serine/threonine kinase that mediates inhibition of apoptosis and stimulation of cell proliferation. There are three

Table 2 Univariable and multivariable analysis of clinicopathological factors and H-score of pAkt against overall survival censored at 10 years

	Univariable analysis		Multivariable analysis	
Clinicopathological factors	**HR (95% CI)**	**p Value**	**HR (95% CI)**	**p Value**
Age	1.04 (1.02 to 1.05)	<0.001	1.05 (1.03 to 1.07)	<0.001
Gender				
Male	1.00		1.00	
Female	0.63 (0.47 to 0.84)	0.002	0.75 (0.55 to 1.03)	0.07
Extent of operations				
Lobectomy	1.00		1.00	
Pneumonectomy	1.64 (1.20 to 2.24)	0.002	2.11 (1.48 to 3.01)	<0.001
Tumour size (cm)				
≤5	1.00		1.00	
5.1–7	1.00 (0.75 to 1.35)	1.0	0.87 (0.64 to 1.18)	0.4
>7	1.18 (0.77 to 1.83)	0.5	1.04 (0.66 to 1.64)	0.9
Histopathology				
Squamous cell carcinoma	1.00		1.00	
Adenocarcinoma	0.87 (0.67 to 1.14)	0.3	1.16 (0.86 to 1.57)	0.3
Large cell carcinoma	0.93 (0.64 to 1.33)	0.7	0.87 (0.52 to 1.45)	0.6
Mixed/others	1.74 (0.89 to 3.44)	0.1	1.58 (0.75 to 3.30)	0.3
Histological grade				
Well differentiated	1.00		1.00	
Moderately differentiated	2.63 (1.23 to 5.64)	0.01	1.73 (0.78 to 3.80)	0.2
Poorly differentiated	2.54 (1.18 to 5.47)	0.02	2.02 (0.90 to 4.50)	0.09
Undifferentiated	3.68 (1.47 to 9.21)	0.006	3.18 (1.07 to 9.44)	0.04
Not reported	2.41 (1.05 to 5.52)	0.04	1.81 (0.72 to 4.57)	0.2
Visceral pleural invasion				
No	1.00		1.00	
Yes	1.27 (0.98 to 1.65)	0.07	1.63 (1.24 to 2.15)	<0.001
Cytoplasmic and nuclear pAkt expression				
High cytoplasmic and low nuclear	1.00		1.00	
Low cytoplasmic and low nuclear	1.76 (1.16 to 2.67)	0.008	1.73 (1.13 to 2.64)	0.01
High cytoplasmic and high nuclear	2.20 (1.24 to 3.90)	0.007	2.30 (1.28 to 4.12)	0.005
Low cytoplasmic and high nuclear	3.59 (1.72 to 7.50)	0.001	2.86 (1.35 to 6.04)	0.006

mammalian isoforms: Akt1/PKB-α, Akt2/PKB-β and Akt3/PKB-γ. It is activated by the binding of ligands, resulting in the phosphorylation of intracellular kinases and the activation of signalling pathways in the cytoplasm. Therefore, it is not surprising to find pAkt expression localised in the cytoplasm. Studies have demonstrated the presence of pAkt in the nucleus suggesting translocation of pAkt from cytoplasm to nucleus[29 30] or, alternatively, the phosphorylation of Akt within the nucleus of cancer cells due to translocation of activated phosphoinositide-dependent kinase 1 from the cytoplasm to the nucleus.[31] The exact mechanism of how pAkt translocates from cytoplasm to nucleus and its function in the nucleus is unclear, but Ahn *et al*[32] suggested that nuclear Akt antagonised apoptosis by phosphorylating transcription factors in the nucleus. It is possible that the nuclear translocation of pAkt results in movement of Akt substrates between cytoplasm and nucleus, which in turn caused cell cycle progression and inhibition of apoptosis.[33] Hence, patients whose cancers had high levels of nuclear pAkt expression had worse prognosis. Only one group of investigators, Shah *et al*,[34] evaluated the relevance of expression at a subcellular level; however, their study was underpowered, utilised a heterogeneous cohort and unsurprisingly failed to demonstrate that the localisation of pAkt had prognostic significance.

Our study showed that patients with cancers that expressed low cytoplasmic pAkt/high nuclear pAkt have a poorer prognosis. However, other investigators have suggested that patients with cancers that expressed pAkt in the nucleus have a better prognosis and responded to targeted therapy better. Cappuzzo *et al*[35] found that patients with advanced NSCLC who were pAkt-positive, as defined by staining in the nucleus, and were treated with geftinib had a better response rate, disease control rate and time to progression than those with pAkt-negative cancers. In breast cancer, Badve *et al*[36] demonstrated that those patients with oestrogen receptor luminal A subtype cancer expressing nuclear pAkt treated with endocrine therapy had a better prognosis. This finding was echoed by Grell *et al*,[37] who showed that patients with HER2-positive metastatic breast cancer that expressed cytoplasmic and nuclear pAkt treated with trastuzumab had a better overall survival. Furthermore, Yang *et al*[38] demonstrated that patients with pAkt-positive breast cancers have a 26% improvement in disease-free survival or a 20% improvement in overall survival, with the sequential addition of paclitaxel to doxorubicin plus cyclophosphamide adjuvant chemotherapy in patients with node-positive early breast cancer. These apparent discrepancies in the outcome of patient with pAkt expression tumours may relate to differences in patient cohort characteristics, for example, breast cancer versus NSCLC, early versus late disease, as well as to variations in methodology and antibody selection. Further studies of pAkt function may provide insights into its biological effects that clarify some of these seeming contradictions.

In conclusion, cytoplasmic/nuclear pAkt expression is an independent predictor of outcome in stage IB NSCLC and may aid in the selection of high-risk stage IB patients. Although it is beyond the scope of this study, it warrants further investigation to see whether high-risk patients would benefit from adjuvant chemotherapy by examining pAkt expression in adjuvant chemotherapy studies.

Take home messages

- Numerous molecular prognostic markers have been examined in non-small cell lung cancer, but to date none have moved into routine clinical practice.
- Level of expression of pAkt in the cytoplasm and nucleus is an independent prognostic factor that may help to select patients with high-risk disease.
- Further investigation of pAkt expression in adjuvant chemotherapy studies is warranted.

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