Characterization of the PI3K Pathway in Non-small Cell Lung Cancer Cells isolated from Pleural Effusions

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Key words

NSCLC, PI3K, S6K, AKT, GSK3β

Word count: 3301 (main text including 3 tables; excluding abstract, refs, figs)

Abstract

Objectives

We hypothesised that it was possible to quantify phosphorylation of important nodes in the PI3K pathway in cancer cells isolated from pleural effusions of patients with NSCLC and study their correlation to somatic mutations and clinical outcomes.

Materials and Methods

Cells were immunomagnetically-separated from samples of pleural effusion in patients with NSCLC. p-AKT, p-S6K and p-GSK3ß levels were quantified by ELISA; targeted next generation sequencing was used to characterise mutations in 26 genes.

Results

It was possible to quantify phosphoproteins in cells isolated from 38/43 pleural effusions. There was significant correlation between p-AKT and p-S6K levels; r = 0.85 (95% CI 0.73 – 0.92); p=<0.0001, but not p-AKT and p-GSK3β levels; r = 0.19 (95% CI -0.16-0.5), p= 0.3. A wide range of mutations was described and p-S6K was higher in samples that harbored at least one mutation compared to those that did not; p=0.03. On multivariate analysis, p-S6K levels were significantly associated with poor survival; p <0.01.

Conclusion

Our study has shown a correlation between p-AKT levels and p-S6K, but not GSK3β, suggesting differences in regulation of the distal PI3K pathway by AKT. Higher p-S6K levels were associated with adverse survival, making it a critically important target in NSCLC.

Introduction

Non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancers. In patients with advanced disease, conventional chemotherapy has reached a plateau in efficacy, with a median survival of 8-11 months [1, 2]. Receptor tyrosine kinase inhibitors (RTKs), such as EGFR and ALK inhibitors, can successfully treat approximately 20% of NSCLC patients [3, 4], but the majority of the population have no identified clinically actionable genetic aberrations [5]. There have been advances in drugs modulating immune checkpoints such as anti-programmed cell death-1 (PD-1) antibodies, with drugs active in approximately 20% of patients with squamous NSCLC and those with a high mutation burden [6].

The development of malignant pleural effusions is a common complication of lung cancer observed in approximately 15% of patients at time of diagnosis [7] and up to 50% of patients during the course of the disease [8]. Pleural effusions are often drained to alleviate symptoms and therefore represent a ready source of fresh tumor cells for genotypic and phenotypic studies.

NSCLC is broadly histologically characterized into adenocarcinoma and non-adenocarcinoma (squamous) subtypes. Comprehensive genomic analysis has shown that the phosphatidylinositol 3-kinase (PI3K) pathway is deregulated more frequently in the squamous lung cancer subtype compared with the adenocarcinoma subtype [9, 10]. However, while studying NSCLC in an unstratified manner, the PI3K pathway may be constitutively active in NSCLC as a result of genetic alterations (e.g. *EGFR* mutations, HER and MET overexpression) affecting RTKs proximal to PI3K in the signaling cascade [11-13]. Alterations of substrates along other signaling networks (i.e. RAS) can also stimulate signal transduction through the PI3K axis. Amplification of *PIK3CA* and *AKT* has been described in 5-37%, and 24%-31%, of samples respectively [14-17], while *PIK3CA* and *AKT* mutations are rare in NSCLC patients [9, 10, 16]. *PTEN* mutations are not frequent in NSCLC [18],

while loss of PTEN protein expression, either partial or complete, is frequently observed in lung cancer [17]. NSCLC cell lines and human tumor specimens are often characterized by multiple alterations of two or more members of the PI3K signaling cascade [16, 17]. In addition, PI3K mutations also coexist with other driver mutations, including *EGFR, KRAS, MEK1, BRAF, ALK* [19-21]; PTEN and KRAS mutations have also been described together in NSCLC specimens [22, 23]. A model of "non-redundancy" has been proposed to explain the presence of co-existing mutations along the PI3K pathway cascade [24].

Functional studies, at a protein level, are complimentary to genotypic studies to understand intracellular pathway deregulation and to guide therapeutic strategies. When aberrant expression of PI3K, AKT and PTEN were evaluated in a series of NSCLC specimens, increased AKT activation (measured by levels of p-AKT) was more frequently observed in those tumors showing simultaneous aberrant expression of two or more substrates of the PI3K pathway [16]. Several groups have investigated the activation of the PI3K pathway in NSCLC by focusing on levels of p-AKT as a marker of pathway activation. Results were obtained using immunohistochemical techniques in the majority of cases [25-28], while the prognostic role of p-AKT overexpression still remains unclear [29, 30]. Immunohistochemistry techniques studying phosphoproteins are challenging to quantify accurately, while de-phosphorylation may occur ex vivo before fixation due to the instability of phosphoepitopes [31].

We aimed to study the feasibility of quantifying the phosphorylation of key proteins along the PI3K pathway (p-AKT, p-S6K and p-GSK3ß) of cancer cells from pleural effusions using semi-quantitative ELISA from patients with NSCLC [32-34]. We further investigated the correlation between phosphorylation of AKT and phosphorylation of the downstream substrates p-S6K and p-GSK3β in order to understand signaling patterns within defined sections of the PI3K network. In addition, we conducted exploratory analyses to assess the

relationship of the activation status of the PI3K pathway with genomic alterations and survival.

Materials and Methods

Patients

Patients with a diagnosis of advanced NSCLC undergoing pleural effusion drainage for symptom control or diagnostic purposes were enrolled in the study after obtaining informed consent. The study was conducted at The Royal Marsden and Epsom and St Helier University Hospital. The protocol was approved by the Research Ethics Committee and Institutional Review Board (Committee for Clinical Research, The Royal Marsden and The Institute of Cancer Research, Ref: CCR3654). Patient details were recorded from available patient records. The study only included patients undergoing drainage of pleural effusion for clinical reasons; the number of lines of previous chemotherapy and decisions about post-procedure chemotherapy varied among patients and were independent from this study.

Immunomagnetic Enrichment of NSCLC cells from Pleural Effusion Samples

Two-hundred and fifty millilitres (ml) of pleural effusion samples were collected at the time of chest drainage and processed within one hour from collection to avoid degradation of phosphoproteins. Five-hundred i.u. of unfractionated heparin were added to every 100 ml of fluid at the time of collection. Each pleural effusion sample was divided into 50 ml aliquots and centrifuged at 1000 g at 4 C^0 to initially obtain cell pellets. Each pellet was re-suspended in 5 mL supernatant and incubated with super-paramagnetic particles coated with the monoclonal antibody BerEP4 (Dynabeads® Epithelial Enrich, Life Technology, UK) on a

rotating wheel for 35 minutes at 4 C. Samples were placed on the magnetic tube rack (Life technology, UK) for 3 minute, and the supernatant was aspirated carefully by pipetting and discharge. The purified pellets so obtained were stored at – 80 C° until ELISA and DNA extraction were performed.

ELISA

ELISA was performed using Meso Scale Discovery multiplex arrays (MSD, Gaithersburg, MD) for phospho-p70S6K (p-S6K), phospho-GSK-3β and phospho-AKT; each well of the 96-well plate is pre-coated with capture antibodies against phospho-p70S6K (Thr421/Ser424), phospho-GSK-3β (Ser9) and phospho-AKT (Ser473). Thirty microliters (µl) of each sample containing 15 µg of proteins were loaded in each well of the ELISA plate and results are thus normalized to protein concentration loaded in each well. Each sample was loaded in two adjacent wells to obtain replicates. The plate was analysed on SECTOR 600 Imager (MSD, Gaithersburg, MD) as per the manufacturer's instructions and the results expressed in ECL (electrochemiluminescent) counts, which provides a quantitative measure of each analyte present in the sample.

DNA Extraction and Sequencing

DNA was extracted from purified NSCLC cell pellets (Qiagen, Manchester, UK) and sequenced using a MiSeq sequencer (Illumina Inc, CA, USA). The TruSight^{TM} Tumor Sequencing Panel was used, which interrogates mutational hotspots in 174 amplicons of 26 genes, listed as follow: *AKT1, ALK, APC, BRAF, CDH1, CTNNB1, EGFR, ERBB2, FBXW7, FGFR2, FOXL2, GNAQ, GNAS, NRAS, KIT, PDGFRA, TP53, KRAS, PIK3CA,*

MAP2K1, PTEN, MET, SMAD4, MSH6, SRC, STK11. Bioinformatics data analysis was performed by the MiSeq Reporter Software MCS 2.2.0, RTA 1.17.28.0. A research report was then generated for each sample showing only somatic mutations detected with coverage above 500X and a quality score of 100.

Statistical Analysis

The pairwise correlation between levels of phosphorylation of AKT and each of the downstream substrates, S6K and GSK3β, was studied using the Pearson's correlation test, where r ranges from -1 to + 1 and 1 equals perfect correlation, 0 no correlation, -1 perfect inverse correlation. The differences in p-AKT, p-S6K and p-GSK3β between patients who had either *KRAS* mutation, *EGFR* mutation or at least one mutation in key genes, and patients with no mutations were analyzed using non-parametric methods (i.e. Mann-Whitney test), using GraphPad PRISM (v. 6, La Jolla, CA).Cox regression analysis was performed using SPSS (v. 22, IBM SPSS Statistics, IL, USA) to estimate the effect of p-AKT, p-S6K and p-GSK3β on patients' survival; survival was defined as the time between the sample was taken and death. Univariate Cox regression model was planned to initially assess the marginal effect of each factor, not corrected for the effect of other factors. Those variables shown to be significantly associated with survival (i.e. all variables with p-value < 0.2 significance in the univariate analysis) were further evaluated in a multivariate Cox regression model to study the simultaneous effect of multiple independent variables on survival. Hazard ratios were calculated for each parameter as well as 95% confidence intervals. Categorical covariates were compared with a predefined reference category.

Results

Patient Characteristics

Over a 12-month period, 43 patients had pleural effusions tapped and 38 had sufficient protein in immunomagnetically-separated cells to be able to quantify phosphoprotein levels. The demographics, histology and treatments prior the pleural tap are summarised in Table 1.

Activation Status of the PI3K Pathway in NSCLC Cells Enriched from Pleural Effusions

Levels of phosphoproteins were quantified in 38 NSCLC samples (Figure 1A). There was considerable variability between patients in the levels of p-AKT, p-S6K and p-GSK3β measured by ELISA, with a coefficient of variation (CV) of 198%, 183% and 97.5%, respectively.

Correlation Analysis between p-AKT and Downstream Substrates p-S6K and p-GSK3β:

Significant correlation between levels of p-AKT and p-S6K was observed; r = 0.85 (95% CI 0.73 – 0.92), p<0.0001. However, there was no significant correlation between levels of p-AKT and p-GSK3ß r = 0.19 (95% CI -0.16-0.5), p = 0.3 (Figure 1B).

Correlation between p-AKT, p-S6K, p-GSK3ß and Somatic Mutations in NSCLC

Table 2 summarizes the details of the 17 mutations detected, the most common being KRAS in 11% (4/38) of patients. Levels of p-AKT, p-S6K and p-GSK3β were compared between patients exhibiting at least one mutation (i.e. *EGFR, BRAF, KRAS, MEK1, PTEN, PI3K, and SMAD4*) and those with no mutation. There were higher levels of p-AKT in samples with mutations compared to those that did not, however this was not significant 74.45 (107.4) vs 12.7 (21.1); p = 0.09 (Figure 2A). Interestingly, significantly higher levels of p-S6K was seen in samples with mutations compared to those with no mutations 168 (sd 241.3) vs 45.7 (sd

69.3); (p = 0.03) (Figure 2B). There was no significant difference between levels of p-GSK3ß amongst samples with mutations or not 586.6 (439.4) vs 618.2 (sd 633.5); p = 0.69 (Figure 2C). There was no significant difference in the levels of p-AKT, p-S6K and p-GSKβ between groups of patients with individual mutations (data not shown).

Correlation between p-AKT, p-S6K, p-GSK3ß and Survival: Survival Analysis

At the time of this analysis 10 out of 38 patients included in the ELISA analysis were still alive. The median survival from diagnosis/ relapse was 11.5 months, reflecting the survival of patients with advanced NSCLC [2] while the median survival from chest drain was 3.8 months. The relationship between p-AKT, p-S6K and p-GSK3β and survival was investigated in exploratory analyses using the Cox regression method. Survival was calculated from the time of chest drain until death or last follow-up. Patients' clinic-pathological characteristics, such as ECOG performance status (PS), age, gender, histology and *KRAS* status were identified as covariates potentially affecting prognosis. Data relating to qualitative and quantitative aspects of smoking history were not available and were not analysed. In univariate analysis for patients' clinic-pathological characteristics, poor performance status, sites of metastases (liver and brain metastases versus others), histology sup-type (squamous cell carcinoma and poorly differentiated carcinoma versus adenocarcinoma) and *KRAS* mutation status were found to be negatively associated with survival. Age and gender were not prognostic in this population. When studying p-AKT, p-S6K and GSK3β, quantified by ELISA and used as continuous variables, higher levels of p-AKT and p-S6K, but not p-GSK3β, were associated with higher risk of death by univariate analysis (Table 3A). When p-AKT and p-S6K were incorporated into a multivariate model together with patients' clinic-pathological characteristics, p-S6K maintained independent and significant association with shorter survival (Table 3B). Among patients' clinical

characteristics, ECOG PS and sites of metastases also maintained their prognostic significance.

Discussion

We have shown for the first time that it is possible to quantify activation of a defined segment of the PI3K signaling network (AKT-GSK3ß-S6K) from cells isolated from pleural effusions of patients with NSCLC. Our approach used immunomagnetic separation of cells with EpCAM expression, a method that has been used previously to isolate circulating tumor cells [33] and separate tumor cells from pleural, pericardial and ascetic effusions [32, 34]. Our results are thus based on EpCAM expressing cells and would miss EpCAM non expressing cancer cells and this could influence the results. EpCAM isolated circulating tumour cells have previously been used in lung cancer research [35, 36] .Other groups have studied activation of intracellular signaling in NSCLC tumor tissue from surgical specimens. A key difference in our study was the use of semi-quantitative ELISA in flash frozen tissue rather than immunohistochemistry on paraffin-embedded tissue [25-28]. Encouraged by the success of this feasibility study, we are further developing multiplex assays to study phosphoproteins in the wider signalling networks outside the PI3K pathway. Because of the advanced nature of patients' disease and the fact that their effusions were often drained for palliative purposes, we did not biopsy patients' primary lung cancer at the time their pleural effusions were being drained. If this had been done, it would have provided valuable insights into the comparisons of signalling between cancer cells in the pleural effusion and the primary tumour.

In previous studies from others groups describing the activation of the PI3K-AKT-m-TOR axis in NSCLC tissue [16, 17, 19, 22], most analyses focused on the phosphorylation of AKT [25, 28, 37-40], while activation of intermediates downstream of AKT remains poorly

defined [16]. Our study has shown that patients with NSCLC have activation of AKT and downstream S6K and GSK3β across multiple specimens. More specifically, we have demonstrated correlation between levels of p-AKT and p-S6K, but not between p-AKT and p-GSK3ß, in NSCLC cells isolated from pleural effusions. This analysis provides novel insights into the PI3K signaling network in NSCLC. GSK3β is a central node over which many different signaling pathways converge. Several kinases phosphorylate GSK3β at the same site as AKT, including p90RSK, a direct substrate of ERK1 on the RAS-RAF-MEK-ERK pathway [41]. Furthermore, GSK3ß is a key intermediate in the WNT signaling cascade and can be activated independently of the PI3K pathway [42]. This independent signaling through GSK3ß may provide a possible mechanism of resistance to PI3K and AKT inhibitors in NSCLC.

Multiple mutations are known to activate the PI3K pathway in NSCLC [11-18]. By using targeted next generation sequencing in cells isolated from pleural effusions, we described a range of mutations, which is not dissimilar to what has been reported in the literature [10]. However, we did not assess TP53 mutations as we were focusing on signal transduction along the PI3K pathway. In the cohort studied, pleural effusion samples that contained at least one somatic mutation were characterised by higher p-S6K levels compared to those samples that did not harbour any mutation. We did not find any STK11 mutations that are known to lead to activation of m-TOR signaling [43, 44]. The oncogene *ALK* is also known to influence PI3K signaling[45]. We could not analyse *ALK* translocations by FISH because the samples were flash frozen following isolation. We did however sequence *ALK* and no mutations were found.

Our patient cohort was probably too small to demonstrate any significant correlation between levels of phosphoproteins (p-AKT, p-S6K or p-GSK3β) and individual mutations. The

heterogeneity of the mutations described and the lack of a singular activating mutation driving signal transduction in these samples reflects the activation of intracellular signaling in NSCLC and the difficulties in identifying biomarkers that predict response to PI3K, AKT and m-TOR inhibitors.

Our study for the first time found that higher p-S6K levels are associated with poorer survival. The hazard ratio was 1.007, with a confidence interval of 1.003 - 1.011, and although this is statistically significant (p=0.001), the clinical significance is not certain and will need to be confirmed in further studies. In this small cohort with NSCLC, approximately 50% of patients had previous treatment and approximately 50% received subsequent chemotherapy, thus it is not possible to draw any conclusions about the prognostic role of p-S6K or its role in acquired resistance to chemotherapy leading to a worse outcome. Nevertheless, these remain important questions that can be addressed in larger sample sets. Interestingly, p-AKT levels were associated with adverse survival in a univariate but not a multivariate analysis. The sample could be the limiting factor as p-AKT levels have been previously shown to be associated with survival in NSCLC, however, the results are not consistent [25, 27, 30].

Conclusions

We have showed that is possible to characterize signal transduction of the PI3K pathway in NSCLC cells isolated from malignant pleural effusions. Our study has demonstrated that p-AKT levels in NSCLC cells isolated from patients correlate with p-S6K levels, but not GSK3ß, suggesting that S6K signaling is dependent on AKT signaling, while GSK3ß is not tightly regulated by AKT. In addition, p-S6K levels were associated with adverse survival, making S6K an interesting target in NSCLC.

Acknowledgments

The Drug Development Unit of The Institute of Cancer Research and The Royal Marsden is supported in part by a programme grant from Cancer Research UK [Ref: C347/A15403]. Support is also provided by the Experimental Cancer Medicine Centre (programme grant to The ICR) [Ref: C12540/A15573] and the National Institute for Health Research Biomedical Research Centre (jointly awarded to The Royal Marsden and The Institute of Cancer Research). The funding sources had no involvement in the submission of this article to Oncology.

Disclosure Statement

The authors declare no conflict of interests.

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Table 1
Clinical characteristics of patients with evaluable pleural effusion sample included in the study and anti-cancer treatment received

Clinical characteristics	*N*
Number of patients	38
Age (median)	71 yrs (range 38-86 yrs)
Gender	Male 58% (22/38)
	Female 42% (18/43)
Histological subtype:	
- Adenocarcinoma	79% (30/38)
- Squamous carcinoma	18% (7/38)
- Poorly differentiated carcinoma	3% (1/38)
Previous anticancer treatment	47% (18/38)
Subsequent anticancer treatment:	50% (19/38)
- Erlotinib	11/38
- Platinum-based doublets	6/38
- Vinorelbine	4/38
- Docetaxel	2/38

Table 2

Genetic alterations detected in 38 NSCLC pleural effusion samples. Gene, AA mutation: change in the peptide sequence; Cosmic Y/N = indicates if the detected mutation is listed in the COSMIC database

Gene	AA Mutation	N	Cosmic Y/N
BRAF	p.V600E	1	Y
EGFR	p.E746_A750del	2	Y
EGFR	p.E709K	1	Y
EGFR	p.L858R	3	Y
KRAS	p.G12C	4	Y
KRAS	p.G12V	2	Y
KRAS	p.Q61H	1	Y
KRAS	p.G12D	2	Y
MAPK21	p.Q56P	1	Y
MAPK21	p.V85G	1	N
PIK3CA	p.Q546H	1	Y
PIK3CA	p.R108H	1	Y
PTEN	p.G127R	1	Y
PTEN	p.H61Y	1	Y
PTEN	p.S170N	1	Y
PTEN	p.G251V	1	Y
SMAD4	p.I525V	1	N

Table 3A

Univariate Cox regression analysis for p-AKT, p-S6K, p-GSK3β and patients' clinico-pathologic characteristics. HR = Hazard Ratio; CI = Confident Interval; Histology, Others = squamous cell carcinoma/poorly differentiated; Histology, Adk = adenocarcinoma

Covariate	**HR**	**95% CI**	**p-value**
ECOG PS			
0/1 (Reference)	1.000		
2	2.500	(0.9 – 6.7)	0.068
3	13.700	(4.1 – 45.9)	<0.001
Variable overall			<0.001
Gender			
Female (Ref)	1.900	(0.9 – 4.1)	0.107
Male			
Age	1.008	(0.97 – 1.05)	0.661
Sites of metastases			
Others (Ref)	1.000		
Brain/liver	4.500	(1.5 – 13.3)	0.006
Histology			
Others (Ref)	1.000		
Adk	0.300	(0.1 – 0.8)	0.017
KRAS			
Wt (Ref)	1.000		
Mut	0.200	(0.1 – 0.8)	0.017
p-AKT	1.004	(1.000 – 1.008)	0.080
p-S6K	1.004	(1.001 – 1.007)	0.004
p-GSK	1.000	(0.999 – 1.001)	0.704

Table 3B

Multivariate Cox regression analysis: for p-S6K, ECOG PS and sites of metastases maintained significant association with shorter survival.

Covariate	**HR**	**95% CI**	**p-value**
ECOG PS			
0/1 (Reference)	1.000		
2	2.700	(0.961 – 7.390)	0.060
3	23.600	(5.512 – 100.793)	<0.001
Variable overall			<0.001
Sites of metastases			
Others (Ref)	1.000		
Brain/liver	4.651	(1.395 – 15.504)	0.012
p-S6K	1.007	(1.003 – 1.011)	<0.001

LEGENDS

Figure 1

Quantification of p-AKT, p-S6K and p-GSK3ß

(A) Histogram shows results of ELISA analysis for 38 NSCLC patients. Each bar represents the median level of p-AKT^{Ser473}, p-GSK^{Ser9} and p-S6K$^{Thr421/Ser424}$ for each patient studied, error bars represent standard error. The numbers in the x axis represent sample ID for the 38 patients analysed. (B) Scatter plot correlation between p-AKT and p-S6K or p-GSK3β; r= coefficient of correlation; ECL count = electrochemiluminescence count measured by ELISA.

Figure 2

Levels of phosphoproteins in samples with mutations

(A) Levels of p-AKT; (B) Levels of p-S6K; (C) Levels of p-GSK3ß