Application of kinase activity profiles to predict survival and therapeutic strategies in early stage non small cell lung cancer.

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Introduction

The prognosis of patients with non-small cell lung cancer (NSCLC) is poor, even in early stage disease. At present, no good diagnostic tests are available to identify early stage NSCLC patients with poor survival or to select patients that might benefit from specific adjuvant therapy. The aim of the current study was to evaluate the prognostic value of kinase activity profiles in early stage NSCLC patients

Traditional measurement of kinase activity in cell lysates is performed in most cases by Western Blotting and immuno-precipitation. This method enables detection of phosphorylation by specific capture antibodies and generic phospho-antibodies, but is time consuming. It lacks throughput and the ability to analyze large numbers of phosphorylation events. Furthermore, knowledge of the target kinase is a requirement.

At PamGene we have simplified kinase activity assays by miniaturization using PamChip® microarray technology. The feasibility of multiplex protein tyrosine kinase inhibitor profiling for patient classification on a peptide microarray was investigated. Here we show the results for profiling NSCLC tissues in the absence and presence of protein kinase inhibitors.

Methods

A retrospective study was performed using frozen tumor tissue from 14 NSCLC patients (stage IA-IIIA) who underwent a complete surgical resection. Patients were grouped based on their overall survival, i.e. short survival (<24 months, n=6) and long survival (>48 months, n=8). Tissue cryosections were lysed in M-Per buffer supplemented with phosphatase and protease inhibitors. Protein content was determined with the BCA assay. The assay conditions were established using a peptide microarray (Fig. 2) comprising 144 PTK peptide substrates derived from known human phosphorylation sites. 10 mg of protein lysate was used per array in the presence of 400 mM ATP. Signal development in time was recorded for 60 min of incubation. Assays were performed in quadruplicate. PTK phosphorylation profiles were determined in the absence and presence of gefitinib. Signal intensities were quantified using proprietary software. Data were analysed using both univariate and multivariate methods. All peptides on the array were included in the PLS-DA class prediction, whereas a peptide selection based on signal intensity was made for the univariate analysis.

Clinical data

Table 1. Clinical data of the patients.

Number	Gender m = male	Age (years)	Status (1=death)	Overall surv (months)	Relaps (1=yes)	Disease free surv (months)	Histology	T-N-M Stage
vb029	m	65	1	4.1	yes	1.6	Large cell	2-0-0
vb044	m	68	1	5.3	yes	2.0	Squamous	2-1-0
vb004	f	51	1	5.6	yes	2.7	Large cell	3-0-0
vb006	m	77	1	6.2	yes	5.5	Squamous	2-0-0
vb039	m	74	1	8.7	yes	5.8	Adeno	2-0-0
vb001	m	60	1	7.5	yes	6.4	Squamous	2-1-0
vb027	m	55	0	> 95	yes	94.6	Adeno	2-0-0
vb030	m	80	1	100.4	no	100.4	Squamous	2-1-0
vb019	f	37	0	> 101	no	101.8	Large cell	2-1-0
vb012	m	59	0	> 103	no	103.7	Large cell	1-1-0
vb045	m	68	0	> 104	no	104.4	Squamous	3-1-0
vb047	m	60	0	> 108	yes	108.8	Adeno	1-0-0
vb010	f	37	0	> 111	no	111.3	Adeno	2-0-0
vb008	m	64	0	> 122	no	121.8	Squamous	2-0-0

Phosphorylation Profiles of Tumors

Lysates from NSCLC tumors (10 µg protein/array) from long term survivors (> 48 months, n = 8) and short term survivors (< 24 months, n = 6), see Table 1, were incubated in a kinase assay mix on microarrays comprising 144 tyrosine containing peptides. Signals were ATP dependent, increased with lysate concentration and could be specifically inhibited by protein kinase inhibitors. Different inhibitors gave different inhibition profiles.

Signals on all peptides were first normalized to the array average and subsequently per peptide to the average of all signals in the samples without inhibitors. This gave basal phosphorylation profiles. These basal profiles showed no obvious relation with tumor type, survival or tumor stage.

The inhibitor gefitinib was spiked into the lysates. Signals were expressed with respect to the uninhibited control. Fig. 1 shows phosphorylation profiles in the presence of gefitinib. Inhibition is represented by a blue color, increase in signal by red. Univariate analysis (t-testing) revealed a set of 19 peptides that were significantly different between the groups of long term and short term survivors. Multivariate analysis (PLS-DA) confirmed that the two groups may be discriminated between. Double LOOCV resulted in an error rate for classification of 29 %.

This prognostic model is being validated on a larger sample set (n = 50).

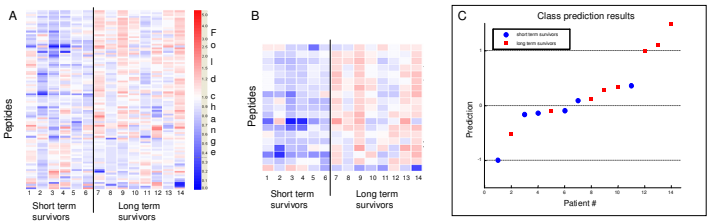

Fig. 1. A. Heat map of fraction of residual activity after addition of kinase inhibitor. Each column represents one patient sample. Samples 1-6 belong to short term survivors, samples 7-14 to long term survivors. Each row represents one peptide. The colour indicates the fold change compared with the uninhibited sample. Red: increase in signal intensity; blue: decrease in signal intensity. **B.** Selection of peptides that are significantly different ($p<0.05$) between both groups. **C**. PLS-DA class prediction based on leave one out cross validation. Each point represents a sample and is colored according to survival group. Samples with prediction < 0 are predicted to be short-term survivors, prediction > 0 are predicted to be long-term survivors.

PamChip® Cell-Based Kinase Assay

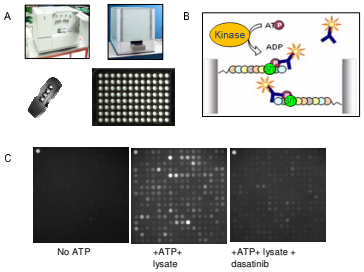

Fig. 2. A. PamStation® 12 and 96 instruments and PamChip®4 and PamChip®96 array plates used for kinase activity profiling. The PamStation® 12 holds 3 PamChip®4 peptide arrays to allow simultaneous processing of 12 arrays. **B**. The reaction takes place on spotted peptides on the array. Peptide phosphorylation through active kinases, or inhibition thereof, can be monitored in lysates of cell lines or tumor tissues by real time fluorescent readout, using a fluorescently labeled phospho-tyrosine specific antibody. **C**. PamChip® arrays after incubation with lysates without ATP, with ATP and with ATP and inhibitor dasatinib.

Conclusions

Phosphorylation profiles can be generated by measuring PTK activity in NSCLC tumor tissue lysates using PamChip® peptide microarrays.

Kinase activity profiles in combination with a kinase targeting drug (gefitinib) can be used to identify early stage NSCLC patients with poor prognosis. The present study introduces a new biomarker discovery platform, which can be applied in drug development and personalized therapy of NSCLC.

Our *in vitro* assay is a promising tool which can serve as a prognostic or as a companion diagnostic test. Response to several kinase inhibitors can be tested on patient biopsy material which will aid in tailoring treatment to each individual patient (Matching Medicine).