Protein tyrosine kinase substrates profiling to detect short-term survivors in early stage lung adenocarcinoma

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Abstract #2366

Introduction:

Adenocarcinoma and squamous cell carcinoma are the most frequent non small cell lung cancer (NSCLC) subtypes and the leading cause of lung cancer-related deaths. Overall survival rate of patients with lung cancer is still very low despite stepwise improvement of existing therapeutic efforts. Although predicted and real outcomes can vary significantly, the assessment of tumor size, lymph node status and the presence of metastasis are currently determining prognosis and treatment modality. Biomarkers with reliable prognostic significance are therefore of utmost importance. Nonetheless, and due to a possible lack of correlation between level of proteins and their corresponding mRNAs, the development of a biomarker screen based directly on enzymatic activities is a promising option enabling the selection of therapeutically targetable enzymes (1, 2).

Protein phosphorylation is an important and ubiquitous post-translational modification in all eukaryotic biological systems with over 500 protein kinase coding motifs identified in human. In general, protein kinases are involved in signal transduction cascades and related kinases may have many common substrates. Besides having common substrates, subtile differences in kinases activities may also determine the relevant *in vivo* signaling pathways. Emerging drugs showing great promise on specific sets of NSCLC patients are targeting protein tyrosine kinase (PTK) activities. Nonetheless, PTK inhibitors proved to be only of temporary relief since the development of secondary resistance leads to high rate of treatment failure. It is therefore becoming important to identify biomarkers with reliable prognostic significance and also, ideally, therapeutically targetable, that may improve both the prognostic power of TNM staging and increase the limited pool of patients that may benefit of the application of the PTK inhibitors for treating lung adenocarcinoma. Here, using the PTK inhibitor gefitinib, we analysed *ex vivo* the PTK enzymatic activities of human lung adenocarcinoma biopsies as potentially important biomarkers in lung cancer biology.

Material and Methods:

Study design and patient characteristics

Established in January 2003, the tumor bank of the Division of Thoracic Surgery at the University Hospital Zurich (UHZ) consists of fresh frozen matched pairs from malignant adenocarcinoma and non-neoplastic lung biopsies. All clinical interventions, follow up treatments, disease-specific survival and outcomes were archived and written consent was required from every participating patient. The clinical characteristics of the 53 patients with early stage 1 and 2 lung adenocarcinoma are summarized in Table 1.

Kinomic profiling of malignant adenocarcinoma and non-neoplastic lung biopsies with multiplexed phosphotyrosylation of peptide substrates

We carried out multiplex and real time ex vivo profiling with lung kinomes of non-neoplastic, adenocarcinoma or adenocarcinoma with 10 uM final gefitinib in two distinct runs of the PamStation®12 and on two independent PTK PamChip®4microarrays (PamGene, 's-Hertogenbosch, The Netherlands).

Statistical analysis

We performed quantitation and quality control investigation with the dedicated BionavigatoR software (version 5.2; PamGene, 's-Hertogenbosch, The Netherlands). The tool PLS-DA (partial least square discriminant analysis) will create class prediction models. Alternatively, the original "training set" samples were also tested by leave-one-out-cross validation. Both analytical approaches resulted in a prediction score called the PamIndex. Finally, for each peptide we applied a correction for the differences of inhibition observed between groups of specimen embedded with or without optimal cutting temperature media (OCT).

Objectives:

Our aim was to document the *ex vivo* multiplexed tyrosine phosphorylation of substrates as a valid molecular approach to create kinome response-signatures of early stage lung adenocarcinoma biopsies and to correlate the prognostic signature obtained with patient survival.

Results 1:

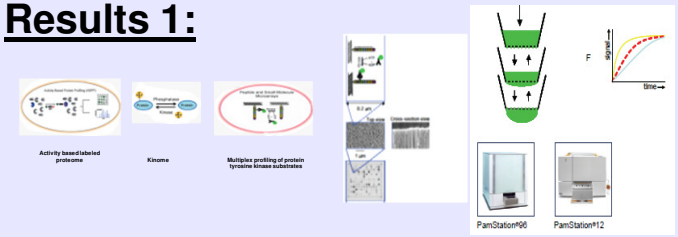

Figure 1: Experimental workflow for PamChip ® Array analysis (modified from www.pamgene.com).

	Stage 1 long term survivors training set (n=10)	Stage 1 short term survivors training set (n=10)	Stage 1 testing set (n=17)	Stage 2 long term survivors training set (n=5)	Stage 2 short term survivors training set (n=5)	Stage 2 testing set (n=6)
TNM Stage Ia	5 (50%)	4 (40%)	5 (29%)	0 (0%)	0 (0%)	0 (0%)
TNM Stage Ib	5 (50%)	6 (60%)	12 (71%)	0 (0%)	0 (0%)	0 (0%)
TNM Stage IIa	0 (0%)	0 (0%)	0 (0%)	2 (40%)	2 (40%)	1 (16%)
TNM Stage IIb	0 (0%)	0 (0%)	0 (0%)	3 (60%)	3 (60%)	5 (84%)
N0	10 (100%)	10 (100%)	17 (100%)	3 (60%)	3 (60%)	3 (50%)
N1	0 (0%)	0 (0%)	0 (0%)	2 (40%)	2 (40%)	3 (50%)
Male	5 (50%)	6 (60%)	8 (47%)	2 (40%)	2 (40%)	3 (50%)
Female	5 (50%)	4 (40%)	9 (53%)	3 (60%)	3 (60%)	3 (50%)
Median age at surgery, years (range)	64 (54-75)	65.5 (46-74)	64 (28-80)	80 (48-88)	67 (52-70)	67.5 (56-77)
Median survival, months (range)	73.9 (62.6-99.3)	34.6 (10.4-52.9)	59.6 (11.7-99.1)	36.9 (30.6-73.5)	12 (8.1-23.3)	29.8 (25.9-98.2)
Locally recurrent disease	1 (10%)	4 (40%)	6 (37%)	2 (40%)	2 (40%)	0 (0%)
Adjuvant therapy	0 (0%)	0 (0%)	2 (12%)	1 (20%)	2 (40%)	3 (50%)
Metastatic disease	0 (0%)	10 (100%)	2 (13%)	2 (40%)	5 (100%)	0 (0%)
Death	0 (0%)	10 (100%)	5 (29%)	4 (80%)	5 (100%)	0 (0%)

Table 1: Characteristics of participating individuals.

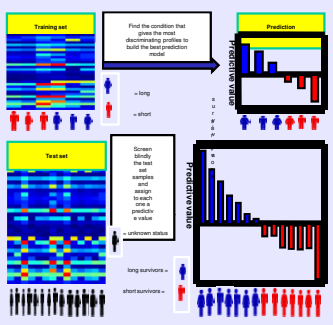

Figure 2: Supervised classification designed to build the best prediction model (modified from www.pamgene.com).

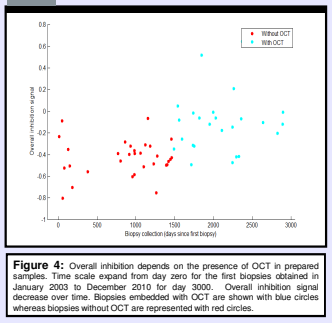

Figure 4: Overall inhibition depends on the presence of OCT in prepared samples. Time scale expand from day zero for the first biopsies obtained in January 2003 to December 2010 for day 3000. Overall inhibition signal decrease over time. Biopsies embedded with OCT are shown with blue circles whereas biopsies without OCT are represented with red circles.

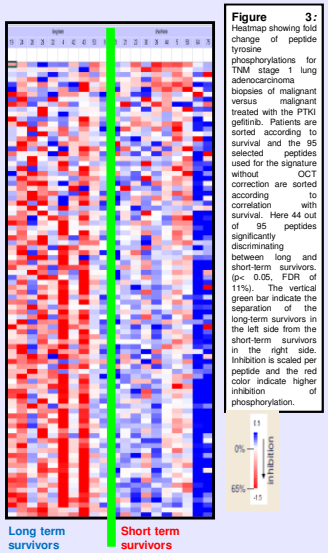

Figure 3: Heatmap showing fold change of peptide tyrosine phosphorylations for TNM stage 1 lung adenocarcinoma biopsies of malignant versus malignant treated with the PTKI gefitinib. Patients are sorted according to survival and the 95 selected peptides used for the signature without OCT correction are sorted according to correlation with survival. Here 44 out of 95 peptides significantly discriminating between long and short-term survivors. (p< 0.05, FDR of 11%). The vertical green bar indicate the separation of the long-term survivors in the left side from the short-term survivors in the right side. Inhibition is scaled per peptide and the red color indicate higher inhibition of phosphorylation.

Results 2:

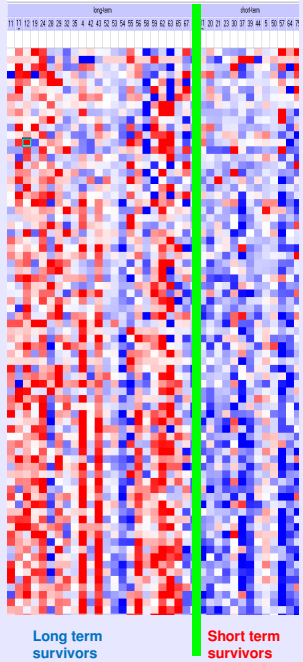

Figure 5: Heatmap showing fold change of peptide tyrosine phosphorylations for TNM stage 1 lung adenocarcinoma patients sorted according to survival. The Log2-transformed ratio of phosphorylation for the biopsies of malignant versus malignant treated with the PTKI gefitinib of the 76 selected peptides used for the signature are sorted according to correlation with patient survival. Inhibition is scaled per peptide and the red color indicate higher inhibition of phosphorylation. The vertical green bar indicate the separation of the long-term survivors in the left side from the short-term survivors in the right side.

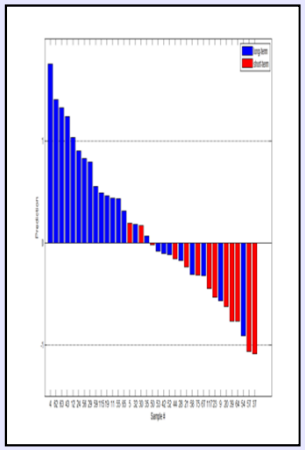

Figure 6: TNM stage 1 lung adenocarcinoma PLS-DA class prediction result performed with the 76 selected peptides. Prediction performance was tested using 10-fold cross-validation for 37 samples tested to obtain here 73% correct prediction. Prediction performance is greater than zero the samples are allocated to the long-term survivors groups or to the short-term survivors when prediction performance smaller than zero. Prediction performance which is further away from the decision boundary set at 0 is less likely to really belong to the opposite group.

Conclusions:

Protein phosphorylation is an important and ubiquitous post-translational modification in eukaryotic biological systems. In this project we screened for tyrosine kinase activities on stage I and stage II adenocarcinoma biopsies in the presence of the the PTKI gefitinib and **we present the feasibility in biopsies of stage I adenocarcinoma to discriminate between long-term and short-term survivors.** We created a 76-point 'response-signature' for each patient's kinome. For the malignant versus the malignant treated with the PTKI gefitinib biopsies, the response signatures presented in the form of an heatmap showed that the long-term survivors had a response signature indicating a strong inhibitory effect of the PTKI gefitinib whereas the short-term survivors cohort was mainly classified with a response signature indicating a weak inhibitory effect at equivalent concentration of the PTKI gefitinib. **When we characterized the 76-point "response-signature" we detected 26 peptides substrates significantly more inhibited with the kinomes of the long-term survivors than with the kinomes of the short-term survivors.** Each of the activity profiles analysed may be more appropriate for a specific type of tumor or patients characteristics. With the idea to obtain a better discrimination in the samples analysed we used here the PTKI gefitinib in the assay as a tool to create the inhibition profiles. The reasons for that are mainly technical and biological. The technical advantage to use an inhibition profile over a non-neoplastic profile is that the malignant profile can be used as a baseline over the inhibited malignant profile. The biological advantage is that some inhibitors may be more efficient on a signaling pathway which is important for the discrimination between samples phenotypes (i.e. long-term versus short-term survivors). In the case of our study the PTKI gefitinib is already described in the clinic as a treatment for adenocarcinoma. **We also observed that the long-term survivors and short-term survivors were properly discriminated in the two distinct types of samples, the OCT and non-OCT groups.** We could correct for those inhibitory effect of OCT after application of a correction showing that our model would probably be best implemented after standardization of frozen tissue biopsy storage **protocols. Nonetheless, we obtained about 73% of the samples correctly** predicted after OCT correction with a 10 fold cross validation PLS-DA analysis based on the 76-point "response-signature" of the pooled TNM stage I lung adenocarcinoma cohort**. Regarding the 26 peptides substrates significantly more inhibited in the kinomes of the long-term survivors,** a biological and mechanistical interpretation of the observed differences is still of interest**.**

References:

1. Wiedl T, Arni S, Roschitzki B, Grossmann J, Collaud S, Soltermann A, Hillinger S, Aebersold R, Weder W. Activity-based proteomics: Identification of ABHD11 and ESD activities as potential biomarkers for human lung adenocarcinoma. J Proteomics 2011; 74: 1884-1894.

2. Wiedl T, Collaud S, Hillinger S, Arni S, Burgess C, Kroll W, Schraml P, Soltermann A, Moch H, Weder W. KRAS Mutation Is Associated with Elevated Myeloblastin Activity in Human Lung Adenocarcinoma. Cancer Genomics Proteomics 2012 ; 9: 51-54.

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