Discovery of a phospho-signature predicting response to dasatinib in non-small cell lung cancer

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Introduction
Targeted drugs are less toxic than traditional chemotherapeutics; however, the proportion of patients that benefit from these drugs is often smaller. A marker that confidently predicts the patient’s response to a specific therapy would allow individual therapy selection most likely to benefit the patient.

Measuring protein phosphorylation levels enables monitoring of over-expression and repression of disease-specific signal pathways. Recent advances in mass spectrometry(MS)-based proteomics allow for the quantitative analysis of phosphorylation events in a global and unbiased manner.

Dasatinib (Sprycel®, BMS) is a multi-kinase inhibitor targeting Bcr-Abl, the Src-kinase family, c-KIT, epidermal growth factor receptor and PDGFRB [1,2]. It is currently approved for chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukaemia. Recently, dasatinib was clinically evaluated in patients with advanced NSCLC. Dasatinib had modest clinical activity, with only one partial response and twelve stable diseases among thirty patients. Neither Src family kinase activation nor EGFR or Kras mutations could predict the response to dasatinib [3]. We therefore applied global quantitative phosphoproteomics to identify a signature of phosphorylation sites that predicts the response to dasatinib in NSCLC cell lines.

Methods
Cell lines
• Selection of 19 NSCLC cell lines with known sensitivity to dasatinib (according to [4] and in-house experiments)
• Classification into sensitive (IC50 <1µM) and resistant (IC50 >1µM) based on CellTiter-Glo® (Promega) viability assays [11] sensitive and 8 resistant cell lines.

Phosphoproteomics workflow
• Isotopic labeling (Arg9/10lys or Arg2/3lys) of cell lines using stable isotope labeling by amino acid in cell culture (SILAC) [5].
• Mixture of protein lysates of 16 randomly selected cell lines served as spike-in standard (super-SILAC) [6] allows accurate cross-sample comparison.
• Application of global, quantitative phosphoproteomics workflow using strong cation exchange chromatography-graphitization (SCX) and immobilized metal ion affinity chromatography (IMAC) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (see Figure 3)
• Processing of raw data with MaxQuant [7].

Significance analysis
• Wilcoxon rank-sum test with subsequent Benjamini-Hochberg FDR correction was used to find differentially phosphorylated sites.
• Functional enrichment of KEGG pathways and Gene Ontology (GO) terms was performed with FatScan [8].
• Significantly regulated sub-networks were detected with the SubExtractor algorithm [9], which combines local as well as topological information (i.e. differential regulation and protein interaction information from STRING, respectively).

Phospho-signature identification (s. Figure 2)
• Robust ensemble feature selection [10] based on the Wilcoxon rank-sum test;
• Linear support vector machine (SVM);
• Estimation of prediction accuracy with a leave-one-out cross validation (LOOCV) procedure.

Results
Phosphoproteomic profiling revealed differentially phosphorylated proteins
• 37,747 phosphoproteins were identified in the profiled cell lines;
• 88% of all quantifications had a cell line to Super-SILAC ratio <4-fold, which allowed for accurate quantification of phosphorylation changes between cell lines;
• From the 37,747 identified phosphorylation sites, 25,020 were determined as class-I sites, i.e. sites that could be identified with high localization confidence.
• Distribution of the phosphorylated residues were: serine 83.2 %, threonine 15.3 %, tyrosine 1.5 %.
• Only signifi-cantly differentially phosphorylated (FDR<10%);
• Most of the regulated sites (95%) were stronger phosphorylated in the sensitive cell lines;
• Functional enrichment analysis revealed that the KEGG pathways Regulation of actin cytoskeleton as well as 40 GO terms were significantly enriched (FDR<5%). Among them were kinase activity, signal transduction, Rho protein signal transduction, cytoskeletal protein binding and actin binding).
• SubExtractor revealed a significantly regulated subnetwork (FDR<5%) centered around the EGFR receptor comprising many proteins involved in cell adhesion and actin cytoskeletal organization (e.g. JUN, CTNNAL, CTNNB3, EPHAZ, BAIAP2, ITGB4 and PLEC1 – see Figure 3).

The phospho-signature was validated in breast cancer cells
• To test whether the phospho-signature is also applicable to other cancer types, we selected 3 sensitive and 3 resistant breast cancer cell lines;
• 5 of the 6 breast cancer cell lines could be classified correctly (Figure 4B); only one resistant sample was wrongly predicted to be sensitive (MDA-MB-468);

Integrin 4α protein expression
• Analysis of unmodified peptides indicate that the measured differences on ITGB4 between sensitive and resistant cell lines on the phosphoproteome level are likely to be due to a difference on the protein expression level (median difference >10-fold).
• Additional experiments to confirm this outcome are currently conducted in-house;
• To investigate the potential clinical relevance of an ITGB4 protein expression marker, we looked at the immunohistochemical staining of lung and breast cancer tissues in the Human Protein Atlas [11]. 54% of the tissue samples showed at least weak staining and 46% no staining (Figure 7), indicating that ITGB4 is indeed differentially expressed in these cancer tissues.

Conclusions
• Identification of response prediction marker from global and unbiased quantitative phosphoproteomics experiments in a preclinical setting is possible.
• The phospho-signature was highly predictive for the sensitivity to treatment with dasatinib in NSCLC as well as breast cancer cell lines.
• The final signature consists of 12 phosphorylated sites on 9 different proteins (see Table 1 and Figure 5);
• These 12 phosphorylations are candidate biomarkers for predicting response in solid tumors to dasatinib and potentially to other Src family kinase inhibitors.
• Analysis of non-phosphorylated peptides showed that the protein expression of ITGB4 is likely to be predictive for sensitivity to dasatinib treatment as well.
• Further experiments to confirm ITGB4 as proteomic response prediction marker are currently ongoing in-house