Multiplex protein tyrosine kinase activity profiling of breast cancer cell lines and tumors tissues on a peptide microarray.

Riet Hilhorst*, Arzu Umar#, Leonie van Houten#, Liesbeth Houkes*, John A. Foekens#, Rik de Wijn*, John W. M. Martens# & Rob Ruijtenbeek*
*PamGene International BV, Nieuwstraat 30, 5211 NL 's-Hertogenbosch, The Netherlands. Phone +31736158076, rhilhorst@pamgene.com, www.pamgene.com
Erasmus Medical Center, Josephine Nefkens Institute, Department of Medical Oncology, Rotterdam, The Netherlands.

Abstract

Prediction of the success of treatment with a particular drug prior to treatment is a holy grail in medicine. It requires the tumor to be tested for the presence of biomarkers, or even more elegantly, for the response to the drug itself. With the advent of protein kinase inhibitors as therapy, and the ability to investigate the effect of protein kinase inhibitors in a tumor lysate on a peptide microarray, this approach becomes feasible.
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, is time consuming and 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 on a peptide microarray was investigated. Here we show the results for profiling breast cancer cell lines in the absence and presence of protein kinase inhibitors.

Methods

10 breast cancer cell lines were grown until confluent. ER, PR, HER2, EGFR status was determined by immunohistochemistry. Protein lysates from 10 different breast cancer cell lines and a squamus carcinoma cell line were prepared with M-PER lysis buffer in the presence of appropriate protease and phosphatase inhibitors. Protein content was determined with the BCA assay. The assay conditions were established using a peptide microarray (Fig. 1) comprising 256 PTK peptide substrates derived from known human phosphorylation sites. 10 µg of protein lysate was used per array in the presence of 400 µM ATP. Signal development in time was recorded for 60 min of incubation. Assays were performed in quadruplicate. PTK phosphorylation profiles were determined for the breast cancer cell lines in the absence and presence of 0.5 and 5 µM PTK inhibitors dasatinib, erlotinib, gefitinib, imatinib and lapatinib Signal intensities were quantified using proprietary software. Only peptides with a signal above a threshold determined based on the cross gene error model were retained in the analysis.

PamChip® Cell-Based Kinase Assay

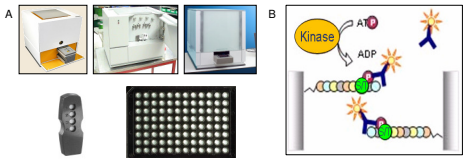

Fig. 1. **A**. PamStation®4, 12 and 96 instruments and PamChip®4 and PamChip®96 array plates used for kinase activity profiling. **B**. The reaction takes place on spotted peptides on the array. Peptide phosphorylation through active kinases, or inhibition thereof, can be monitored in lysates of cell lines or tumor tissues by real time fluorescent readout, using a fluorescently labeled phospho-tyrosine specific antibody

Assay Optimization for Cell Lysates

Lysates from ten breast cancer cell lines were incubated in a kinase assay mix on an array comprising 256 peptides. First, it was established that signals were ATP dependent, increased with lysate concentration and could be specifically inhibited by protein kinase inhibitors (Figures 2 and 3). 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. Fig. 4 shows in green the signal intensities that are higher than the average of all cell lines and in blue those lower than the average.
The effect of the inhibitors dasatinib, erlotinib, gefitinib, imatinib and lapatinib is depicted in Figs 5 and 6. Signals are expressed with respect to the uninhibited control. Inhibition is represented by a blue color, stimulation by red.

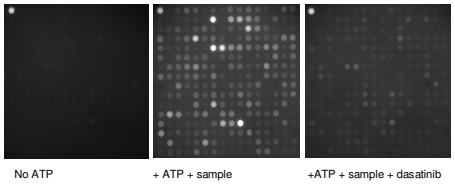

Fig. 2. Images of PamChip® arrays after incubation of the array with a cell lysate in the absence (left) and presence (middle) of ATP and the effect of addition of the inhibitor dasatinib to the incubation mixture (right).

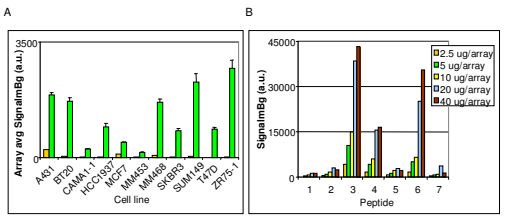

Fig. 3. A. Comparison of average signals per array in the absence (orange) and presence (green) of ATP for lysates from 10 breast cancer cell lines and one squamous carcinoma cell line. B. Signal dependency on the amount of protein (cell line HCC1937) that was applied per array. Data are shown for 7 of the 256 peptides.

Phosphorylation Profiles of Cell Lysates

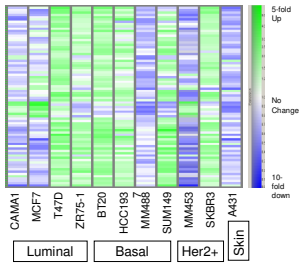

Fig 4. Phosphorylation profile for ten breast cancer and one squamous carcinoma cell line. Each horizontal line represents a peptide. Each column represents a cell lysate. Signals are averaged per array and per peptide.

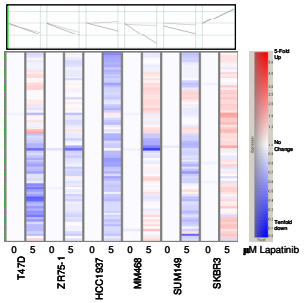

Fig 5. The effect of lapatinib on peptide phosphorylation for six cell lines. Each line represents a peptide. Each column represents a cell lysate without (0) or with (5 µM) lapatinib. Top panel: Effect of lapatinib on the phosphorylation of ERBB2 site Y877 and autophosphorylation site Y1248.

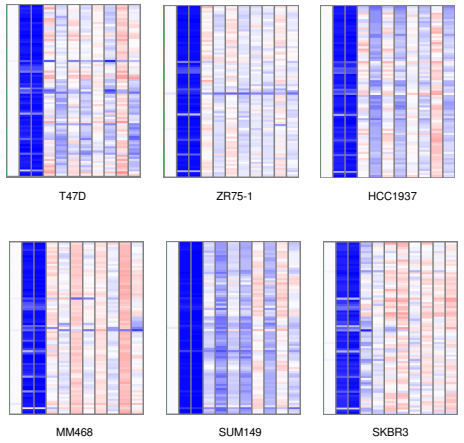

Fig 6. The effect of five inhibitors on peptide phosphorylation for 6 cell lines. Inhibitors from left to right for each cell line: no inhibitor, 0.5 and 5 µM of dasatinib, erlotinib, gefitinib, imatinib and lapatinib. Signals are normalized to the control without inhibitor.

Conclusions

Phosphorylation profiles can be generated by measuring PTK activity in breast cancer cell line and tumor tissue lysates. using PamChip® peptide microarrays. Based on the phosphorylation profiles, cell lines do not cluster according to their basal, luminal and HER2+ subtype.
In addition, the effect of inhibitors on the PTK phosphorylation profile could be studied *in vitro*. Addition of protein kinase inhibitors affected phosphorylation patterns in a specific manner. Notably dasatinib was a very potent inhibitor.
Lysates from freshly frozen breast tumors also gave distinct phosphorylation profiles.
These results show that the effect of inhibitors on kinase activity can be determined in a complex cellular environment (data not shown).
Our *in vitro* assay is a promising tool which, in future, can serve 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.