Two case studies on kinase activity profiling in non-small cell lung carcinoma tumour tissues:

# Identification of prognostic biomarkers and Identification of biomarkers that predict response to erlotinib treatment.

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## Aim

Evaluate the prognostic value of kinase activity profiles and kinase inhibition profiles in early stage NSCLC patients
- for prognosis of long- or short-term survival,
- for predicting erlotinib response.

Reliable diagnostic tests are needed to identify early stage non-small cell lung carcinoma (NSCLC) patients with poor prognosis. Concomitantly there is a clear need for tests that enable the selection of patients who will benefit from targeted therapy with kinase inhibitors. We evaluated kinase activity profiles in two groups of early stage NSCLC, either for prognosis of long- or short-term survival, or for predicting erlotinib response

## Identification of prognostic biomarkers

A retrospective study was performed on two groups of early stage NSCLC patients (stage IA-IIIA) who underwent a complete surgical resection. The groups consisted of short term survivors and long term survivors (survival duration from diagnosis < 24 months (n=22) and > 48 months (n=26). Follow-up was at least 5 years for all patients.

Table 1. Patients characteristics.

| | Short term survivors | | | Long term survivors | | |
|---|---|---|---|---|---|---|
| | Adeno | Squamous | Large Cell | Adeno | Squamous | Large Cell |
| Patient demographics | | | | | | |
| Total number of samples n (relaps) | 7 (4) | 9 (6) | 6 (6) | 9 (3) | 11 (1) | 6 (0) |
| Age, median | 68 | 65.6 | 63.4 | 55.9 | 67.0 | 67.5 |
| Male | 6 (5) | 9 (6) | 4 (4) | 5 (3) | 10 (1) | 5 (0) |
| Female | 1 (1) | 0 | 2 (2) | 3 (0) | 1 (0) | 1 (0) |
| Disease Free Survival, median (months) | 8.1 | 9.0 | 6.0 | 81.3 | 65.1 | 77 |
| Overal survival, Median (months) | 13.6 | 10.7 | 10.9 | 81.3 | 66.4 | 83 |
| Primary tumor TNM stage | | | | | | |
| T1N0M0 | | | | 2 (1) | 1 (0) | 2 (0) |
| T1N1M0 | | 1 (1) | | 1 (0) | 1 (1) | 1 (0) |
| T2N0M0 | 4 (4) | 1 (0) | 3 (3) | 6 (2) | 6 (0) | 1 (0) |
| T2N1M0 | 2 (2) | 3 (3) | | | 2 (0) | 2 (0) |
| T3N0M0 | | 3 (3) | 2 (2) | | | |
| T3N1M0 | | 1 (1) | 1 (1) | | 1 (0) | |
| T4N0M0 | 1 (0) | | | | | |

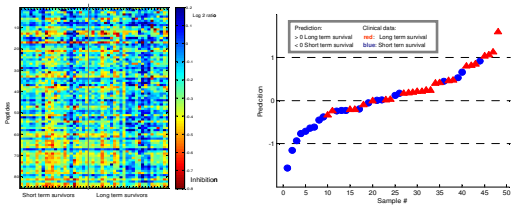


**Fig. 1.** Left. Classification of samples based on kinase inhibition profiles obtained by spiking gefitinib into the tumor lysates. Each horizontal line represents a peptide, each column a sample. Right: PLS-DA class prediction for 22 short term survivors and 26 long term survivors based on leave one 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.

Results
Phosphorylation profiles obtained in the absence of gefitinib did not distinguish between patients with short survival and long survival, whereas ratios of inhibited vs. non inhibited signals resulted in a classifier able to predict survival. Interestingly, the inhibitory effects of gefitinib were stronger in patients with short survival times as compared to patients with long survival times.
Multivariate unsupervised analysis with leave-one-out cross-validation resulted in the correct prediction of outcome for 33 out of 48 patients (71 %)

## Identification of biomarkers that predict the response to erlotinib

Investigations were performed on 14 samples of early stage (stage 1A-IIIA) NSCLC patients included in the M06NEL study. This is an open label, multicenter, non-comparative phase II study.

Table 2. Data for 14 (stage IA-IIIA) NSCLC patients included in the M06NEL study. Operable patients, 18 years and over, with resectable NSCLC (cT1-3, N0-1) were eligible for the study.

| | Responders | Non-responders |
|---|---|---|
| Patients | 6 | 8 |
| Male | 2 | 6 |
| Female | 4 | 2 |
| Tumor Type | | |
| Adenocarcinoma | 5 | 7 |
| Squamous | 0 | 1 |
| Large Cell | 1 | 0 |
| Smoking status | | |
| Never smokers | 2 | 1 |

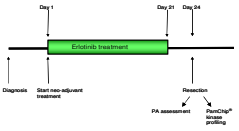


Fig. 2. Clinical study design. Preoperative treatment comprised of 150 mg erlotinib daily for a period of 3 weeks. Surgical resection involved a radical resection of the tumor. Response evaluation to neo-adjuvant treatment was done by histological assessment of the surgical specimens according to Junker et al (3).

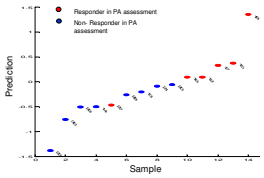


Fig. 3. PLS class prediction for 14 samples based on double leave-one-out cross-validation.

Results
Ratios of inhibited vs. non inhibited signals, were used as input for multivariate unsupervised analysis with leave-one-out cross validation. This resulted in 13 out of 14 (93%) correct predictions, and 100% correct prediction of non-responders. The results are represented as prediction values in Fig 3.
The classification in patients with EGFR or KRAS mutations was coherent but not identical with mutation data (among 6 responders 1 showed EGFR ex19 mutation and among 8 non-responders 1 had EGFR ex 20 (9BP in frame deletion) and 3 had KRAS codon 12 mutations).

## PamChip® Kinase Assay

Fresh frozen tissue cryosections were lysed in M-Per buffer supplemented with phosphatase and protease inhibitors. Kinase activity profiles were determined in the presence and absence of erlotinib or gefitinib on PamChip® arrays comprising 144 tyrosine containing peptide sequences derived from known human phosphorylation sites. Peptide phosphorylation was detected with anti-phosphotyrosine antibodies and monitored in time. Signal intensities per peptide were quantified using proprietary software. $^2$Log Ratio's of inhibited *vs.* non-inhibited signals were used as input for multivariate analysis to classify samples.

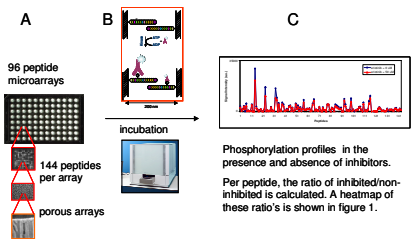


Fig.4. PamChip® peptide microarray technology (see ref. 1-2).

## Conclusion

Using a novel biomarker discovery platform we show that kinase activity profiles of tumour tissue exposed *ex vivo* to a kinase inhibitor can support
- the identification of (early stage) NSCLC patients who are likely to respond to TKI treatment.
- the prognosis for early stage NSCLC patients.

References
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