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Multimodality approach towards individualized non-small cell lung cancer treatment

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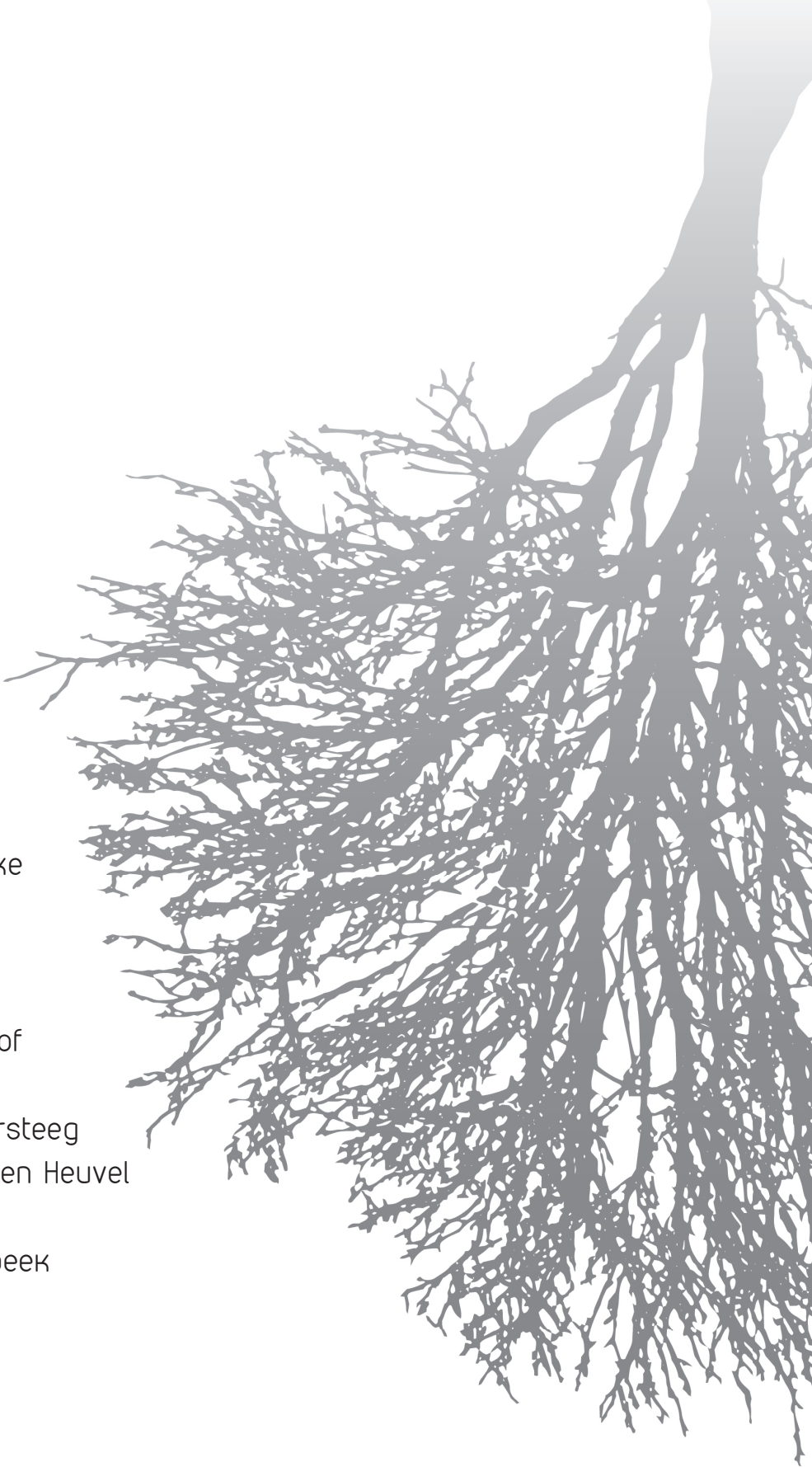
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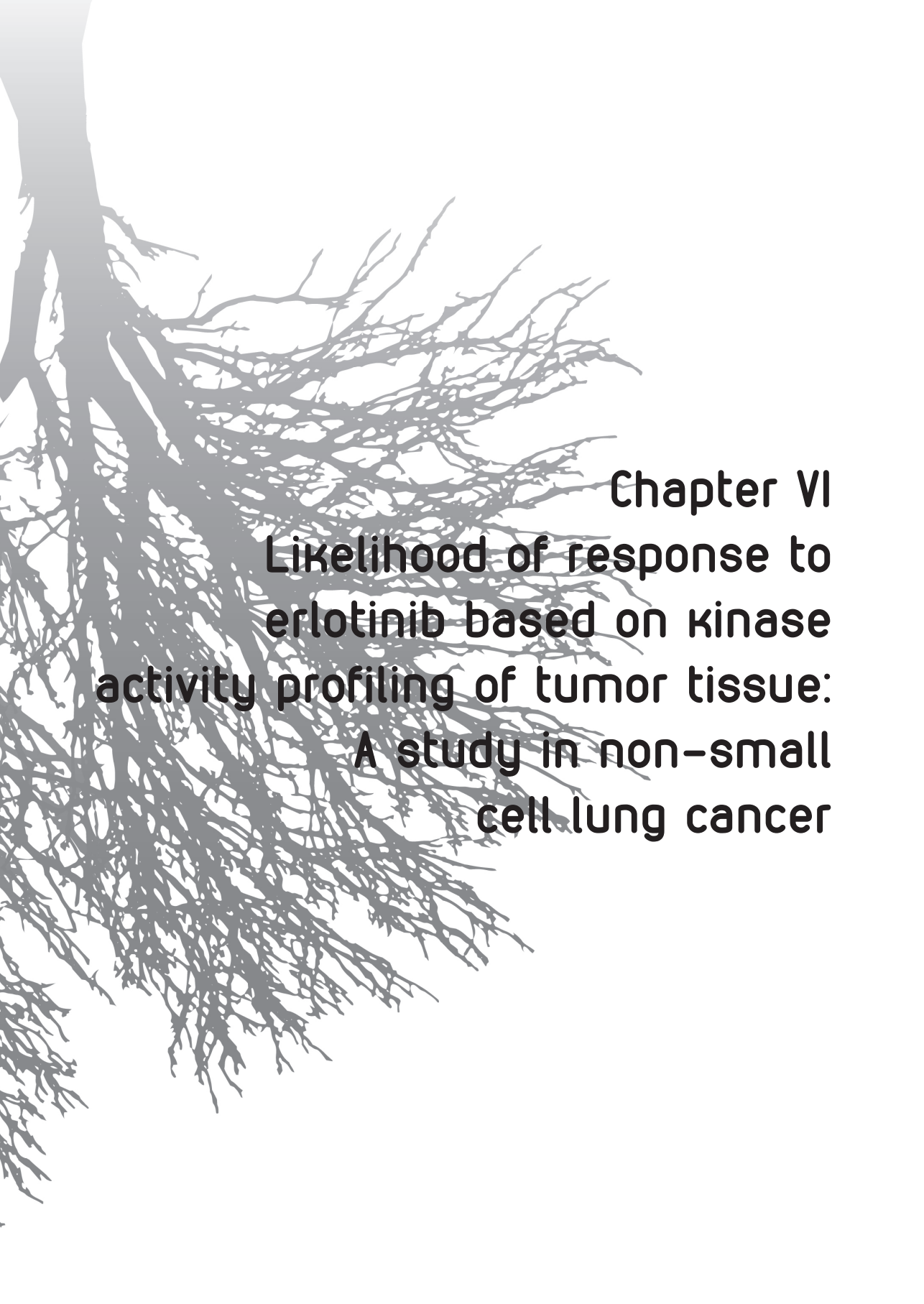
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A grayscale silhouette of a tree branch, showing a thick trunk on the left that branches out into a dense network of thinner, leafless twigs extending towards the right and top of the frame. The background is plain white.

Chapter VI
Likelihood of response to
erlotinib based on kinase
activity profiling of tumor tissue:
A study in non-small
cell lung cancer

ABSTRACT

Background | Patients with Non-Small Cell Lung Cancer (NSCLC) that harbor mutations in the tyrosine kinase domain of Epidermal Growth Factor Receptor (EGFR) have a higher chance of response on Tyrosine Kinase Inhibitors (TKIs). However, a subgroup of patients lacking these mutations may also benefit. The objective was to investigate kinase activity profiles in tumor tissue in the presence and absence of erlotinib and to relate this ex vivo response to clinical response.

Methods | Frozen tumor tissue was obtained from two groups of patients with resectable NSCLC (stage IA-IIIa). One group was untreated, the other group received erlotinib for 21 days before surgical resection. Tissue cryosections were obtained and lysed in a buffer supplemented with phosphatase and protease inhibitors. Kinase activity profiles of the lysates were generated on PamChip® peptide microarrays in the presence and absence of erlotinib, allowing determination of the ex-vivo inhibition of kinase activity. Kinase inhibition profiles for a set of samples (n=16) were used to train a classifier to predict response to erlotinib inhibition, followed by a validation series using blinded test samples (n=15). Clinical response evaluation was based on PET/CT and histopathologic assessment. All specimens were analyzed for EGFR and KRAS mutation status.

Results | A classifier was obtained that distinguished erlotinib responders (n= 13) and non-responders (n=17) in the training set, using a Leave-One-Out Cross Validation and resulted in misclassification of two samples. Application of the classification algorithm to 15 blinded samples from an independent validation set resulted in correct prediction of outcome for 12 samples.

Conclusions | A classifier was established based on kinase inhibition profiles that predicts response to erlotinib. The functional inhibition test at the kinase level may identify patients who respond to a TKI in the absence of an activating mutation.

INTRODUCTION

Survival in patients with non-small cell lung cancer (NSCLC) remains disappointing, even in patients with early stage disease (1). (Neo) adjuvant treatment with radiotherapy or chemotherapy has a limited effect on disease free survival and overall survival (2, 3).

The development of “targeted therapy” has led to a new era of clinical research with promising results. The Epidermal Growth Factor Receptor (EGFR) is expressed in many solid tumors including NSCLC. Inhibition of the tyrosine kinase domain of this receptor by EGFR tyrosine kinase inhibitors (TKIs), such as erlotinib and gefitinib, prevents downstream signalling involved in cell proliferation, angiogenesis, invasion and metastasis. Activating mutations in the EGFR tyrosine kinase domain are associated with increased progression free survival in patients with NSCLC, especially adenocarcinoma (4, 5) and higher response rates to EGFR-targeting drugs (6). Whereas patients harbouring a KRAS mutation have a poorer progression free and overall survival (5).

Erlotinib is a small-molecule EGFR-TKI, registered for the treatment of patients with advanced NSCLC (7, 8). It can be orally administered, and has a relatively favorable toxicity profile, which makes it a potentially appealing drug to use in the neoadjuvant or preoperative setting (9, 10). To select patients with NSCLC who may benefit from EGFR-TKI treatment, several approaches have been used. With selection based on phenotypes (adenocarcinoma, female, non-smoker, Asian ethnicity) a response rate of 30% can be achieved (11, 12). With the availability of tumor tissue, molecular selection based on EGFR mutations can increase the response rate to around 70% (13-15). Sensitivity to TKIs is based on complex signaling cascades of tyrosine and serine/threonine kinase activity. Multiple targets can signal for the same transcription factor within the cascade. Therefore, some patients with EGFR-mutations will not respond to TKI therapy, whereas others can become resistant after some time. Acquired TKI resistance can arise due to secondary mutations in EGFR (e.g. T790M) (16, 17), selection of resistant cells or activation of alternative signaling pathways. In 10-20% of the patients with objective response to a EGFR-TKI, no mutations have been identified in EGFR (4, 13, 18).

Kinase activity measurements on PamChip® peptide microarrays have been performed in several cancer types (19-30). The arrays contain peptides which are phosphorylated by the kinases present in the cell lysates obtained from the tumor. The kinase activity of a sample is measured and results in a phosphorylation profile. Versele et al. showed proof of principle using ex vivo EGFR-TKI testing on non-treated tumor cell lysates to predict “treatment response” in terms of inhibition of proliferation in multiple cell line models (30). Kinase phosphorylation profiling was also used to predict response to preoperative chemoradiotherapy in locally advanced rectal cancer (20, 20, 22, 24). In addition to the phosphorylation profile a functional assay, determining kinase activity profiles of a tumor sample in the absence and presence of

a targeting drug, such as erlotinib, potentially may improve adequate prediction of a patients' response to such a drug (13, 31). Current response prediction models indicate a probability of response based on statistical analysis of a large number of patients sharing a particular tumor property, without taking into account individual functional characteristics. The primary objective of this study was to investigate kinase activity profiles in tumor tissue of patients with NSCLC in the presence and absence of erlotinib and to relate this *ex vivo* response to clinical response. If the effect of adding the drug to the lysate of a patient's tumor reflects clinical response of this individual, this approach could be a further step towards personalized medicine.

METHODS

Patient population

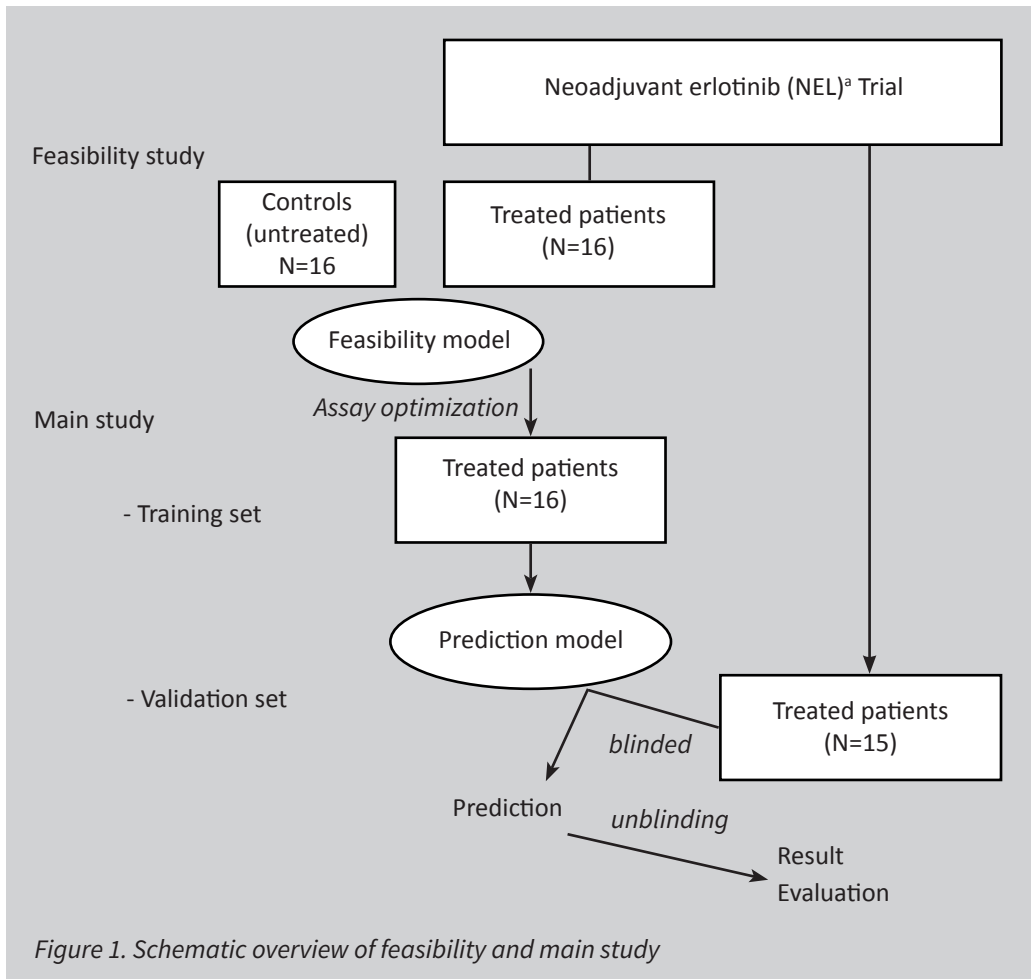
Patients with resectable NSCLC were included in a phase II preoperative trial, receiving neoadjuvant erlotinib daily during 3 weeks until resection (32). Frozen tumor tissue of resectable NSCLC from the NKI-AvL was collected. Patients used erlotinib for a median of 20 days, and discontinued treatment median 3 days before surgical resection. A control group was added with patients who did not receive preoperative treatment, matched with respect to tumor histology, gender and age. A schematic overview is shown in Figure 1. This study was approved by the protocol review board and was performed in accordance with the Helsinki declaration. Informed consent was required for participation.

Patients with newly diagnosed resectable NSCLC – i.e. clinical stage I-II NSCLC, cT1-3 N1-0 - were allowed to enter the study. Inclusion and exclusion criteria have been described by Schaake et al (32), as well as treatment schedule, toxicity and assessment of response. Responders were defined as having metabolic response according to EORTC criteria and more than 50% necrosis with signs of therapy-induced tissue alterations in the resection specimen, non-responders had stable or progressive disease. EGFR and KRAS mutation testing was performed in the certified diagnostic laboratory of the NKI-AvL (33).

Sample preparation

Cryosections of 10 μm thickness were cut from fresh frozen NSCLC resection material. The sections were distributed over 3 vials and stored at -80°C till use. The tumor content was determined by HE staining and ranged from 3 to 100 %.

Tissue cryosections were lysed for 30 min on ice in lysis buffer (M-PER Mammalian Extraction Reagent supplemented with Halt Phosphatase Inhibitor Cocktail and EDTA-free Halt Protease Inhibitor Cocktail (Pierce Biotechnology, Inc., Rockford, IL)). After centrifugation for 15 min at 10000xg at 4°C , the supernatants were aliquoted and snap frozen on dry ice. Protein content of the lysates was determined with micro BCA assay (Pierce Biotechnology, Inc., Rockford, IL), using BSA as reference protein. All experiments were performed using lysate aliquots that had not been thawed before.

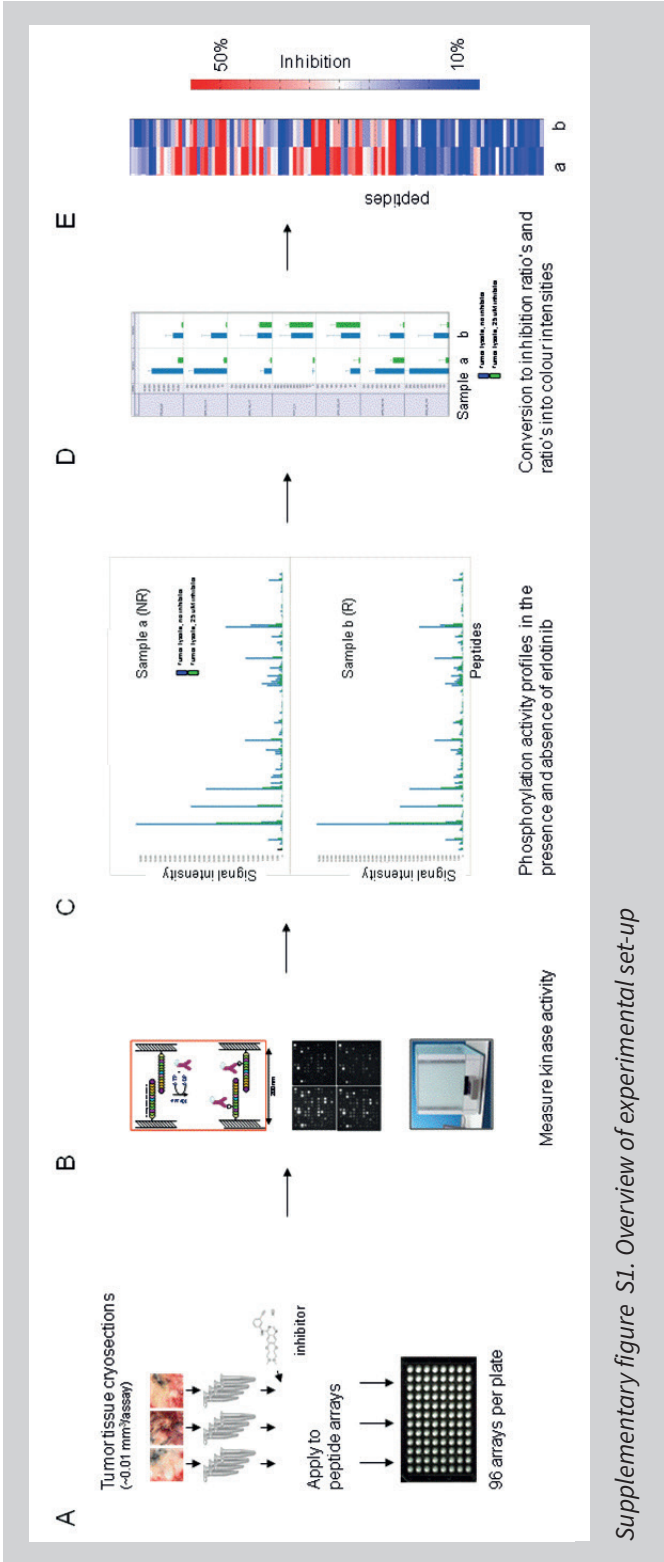


Kinase activity profiling

Incubations of the lysates in the presence and absence of erlotinib and dynamic readings of kinase activity on PamChip® 96 peptide microarrays (#86311, PamGene International BV, 's-Hertogenbosch, The Netherlands) have been performed under conditions as described in (22). A flow chart of the experimental steps is given in Supplemental Fig S1.

Feasibility

The use of post-treatment tumor tissue to predict a likely response to erlotinib treatment is only feasible when the treatment has not significantly altered the properties of the tumor and thus altered the outcome of the inhibition profile. Therefore the untreated samples were added as a control group to the inhibition profile test phase. The feasibility study was performed using samples from one part of patients treated with erlotinib (the training set, n=16) and samples from the untreated control



Supplementary figure S1. Overview of experimental set-up

group (n=16). Patient samples (7 µg of protein per array) were analyzed in quadruplicate without erlotinib, and in duplicate with 5 µM erlotinib (LKT Laboratories, St Paul, MN, USA) in the incubation mixture. The final DMSO concentration was 0.5 % v/v. Patient samples of the training and control set were distributed over 96 microarrays (96 per plate) per run. A basal optimisation was performed.

Training and validation

Before performing the validation experiment, further assay optimization was performed with respect to sample input and erlotinib concentration. Based on those results, the amount of protein used per assay was adjusted according to the mean signal intensity of the 15 peptides with the highest signal, and varied between 2 and 20 µg per array. Samples were normalized for basal signal heights during validation, without inhibition. The erlotinib concentration was increased to 25 µM, compared to 5 µM in the feasibility, as for unexplained reasoning a higher concentration was needed for the same 30% inhibition.

The validation experiment was performed using the samples from the first part of patients, the training set (n=16), and a separate (blinded) validation set (n=15) obtained from the other part of patients enrolled in the neoadjuvant erlotinib study. Patient samples of the training and test sets were randomly distributed over 96 microarrays (96 per plate) per run.

The whole procedure took 2 hours to perform. Clinical response data of the validation set samples were only revealed, after reporting read-out and likely response of the samples to the NKI-AvL.

Data analysis

After visual check of all arrays and grids for spot finding, signals for each peptide were quantified with BioNavigator software (PamGene, 's-Hertogenbosch, The Netherlands). Defective arrays were removed after data inspection. Measurements were performed in duplicate. For each spot on the array, signal intensity after subtraction of local background was calculated and used for further analysis. All data processing and visualizations were performed using Bionavigator and Matlab (R2010B, The Mathworks, Natick, MA)

Data pre-processing for the feasibility experiment

Signals measured at the end of the incubation (end levels, EL) were used for analysis. Data were ²log transformed, hereto a small number of negative values in the data needed to be handled prior to log transformation. It occurred that clear signals were lower than background signals, therefore resulting in a negative signal after subtraction of the background signal. This was done applying an “upward shift” to the data. Hereto the 1% percentile point + 1 of the full data set was subtracted from the data, values remaining < 1 after the shift was set to 1. In order to – largely – remove the subset of peptides that showed weak or absent signals, peptides for which the mean

log transformed signal in the incubation without inhibitor was < 9.551 (${}^2\log 750$) were excluded from the profiles, leaving 79 peptides for further analysis for each sample. For each sample an inhibition profile was calculated by subtracting the ${}^2\log$ value with inhibitor from the ${}^2\log$ value without inhibitor. In formula: for each peptide j in sample i the log-fold-change $L_{ij} = S_{ij}^0 - S_{ij}^1$, was calculated, where S_{ij}^0 and S_{ij}^1 are the log-transformed signals for peptide j in sample i without and with inhibitor added, respectively. The log-fold-change L_{ij} corresponds to the ${}^2\log$ of the ratio of the measured activity signal without and with inhibitor added before log transformation. It is equal to zero for peptides for which no changes occur, and < 0 for peptides for which blocking of the relevant kinase activity occurs in the presence of a blocking agent such as erlotinib.

Class prediction model for the feasibility experiment

Based on the ${}^2\log$ inhibition ratio profiles of the training set a class prediction model was built in Matlab using partial least squares discriminant analysis (PLS-DA), (34) without further selection of discriminative peptides; all peptides were included with differential weights. The performance of the class prediction model was estimated by leave-one-out-cross-validation (LOOCV), ensuring that for each iteration of the cross validation the model was built completely independent of the left out sample (35). For both the training and the test set a model was tested with each of the samples individually left out to test the robustness of response prediction. Application of this class prediction model to a new sample results in a prediction index ("PamIdx"), where $\text{PamIdx} > 0$ means that the sample is predicted to be a responder, a $\text{PamIdx} < 0$ means that the sample is predicted to be a non-responder

Data preprocessing for the validation experiment

For the validation study, inhibition profiles were calculated in the same way as for the feasibility experiment. In this case a larger set of 121 peptides was retained for further analysis after applying the signal threshold. It was found that the validation set samples as a whole showed a lower signal and inhibition level than the training set samples. This systematic difference between the training and validation set was accounted for by scaling each included peptides to zero mean and unit variance in the training and validation set separately, prior to the class prediction analysis.

Class prediction model for the validation experiment

In the main study the class prediction model was built as described for the feasibility experiment using a training set and validating it on a validation set. Prediction performance was evaluated by LOOCV of the training set (as described for the feasibility experiment) and by applying the prediction model that was built using the training set, to the data obtained from the validation set. The clinical response data of the validation set were only revealed after reporting the predicted response to the NKI-AvL.

Table 1. Patient characteristics

		Erlotinib						Untreated	
		Training set		Test set = 15		Total =31		Control group =16	
		N=16							
		Count	Mean	Count	Mean	Count	Mean	Count	Mean
Sex	Male	8		6		14		6	
	Female	8		9		17		10	
Age at diagnoses			60		63		62		57
Histology	Large cell undiff.	1		4		5		1	
	Squamouscell ca	2		4		6		2	
	Adeno carcinoma	13		6		19		13	
	Broncheo alveolair ca	0		0		0		0	
	A-typical carcinoid	0		1		1		0	
Smoking status	Never	6		3		9		1	
	Former/Current	7/3		7/5		14/8		12/2	
	Packyears		33		43		38		23
EGFR mutated	No	14		12		26		15	
	Yes	2		3		4		1	
KRAS mutated	No	12		13		25		10	
	Yes	3		2		5		6	
	Not performed	1		0		1		0	
Days erlotinib			20		19		19		0
Follow up months			34		25		30		40

RESULTS

Patient characteristics

The patient population reported was enrolled between January 2007 and May 2010, the diagnostic evaluation, treatment and follow-up were performed in the NKI-AvL. For this analysis 31 patients received neoadjuvant erlotinib and 16 untreated control patients were analysed. Table 1 summarizes the demographic and tumor characteristics of the treated and untreated patient groups.

Feasibility study

Inhibition profiles of the training and the control set (as illustrated in Figure S1) were compared (Figure S2) to evaluate whether the treatment of the patients led to significant differences between the groups. Univariate analysis of the profiles did not yield any peptides that differed significantly between the treated and untreated group, showing that it was feasible to use the (post-treatment) samples of the training set for this study.

The $^2\log$ inhibition ratio profiles of the training set ($n=16$) were used to test the feasibility of building a classifier to distinguish responding from non-responding, using partial least square discriminant analysis (PLS-DA). Two samples prevented the building of a useful model. For one of these patients (T-16), who had concomitant NSCLC & CLL, tumor material from a metastatic lymph node was used in the test. The other sample (T10) was an atypical case, a squamous cell carcinoma with response to erlotinib. Exclusion of these samples resulted in a model that was validated with Leave One Out Cross Validation (21). These data showed that it was feasible to distinguish responders from non-responders. The assay conditions were further optimized with respect to sample input (amount) and inhibitor concentration to allow an optimal separation of responders and non-responders.

Building a model with the training set

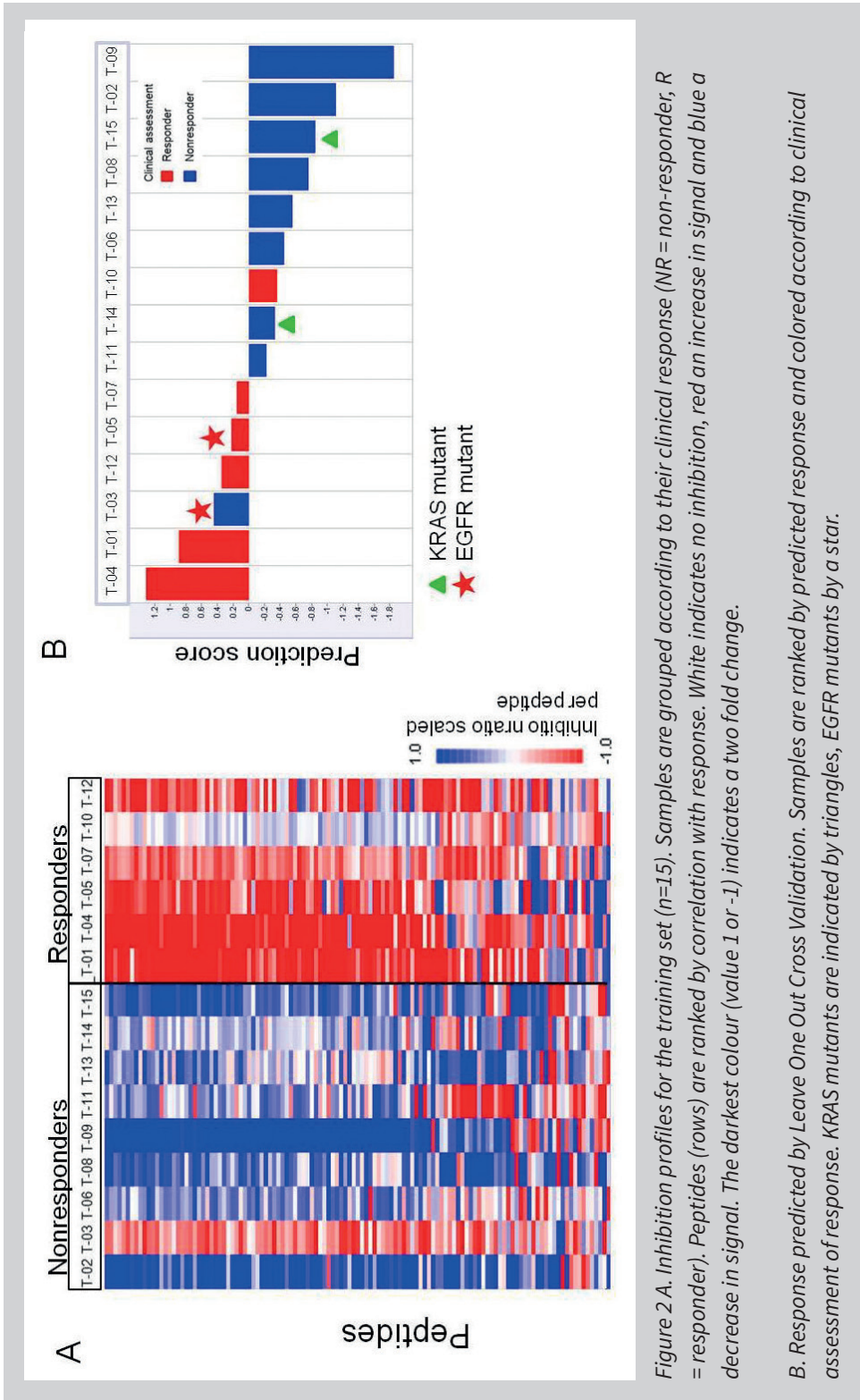
The inhibition profiles of the training and validation set were determined. Based on the $^2\log$ inhibition ratio profiles of the training set (Figure 2A), a classifier was built. Sample T-16 distorted the model and had to be removed. Sample T-10 could be retained, leaving 15 samples in the Training set. The performance of the classifier in the training set was checked by LOOCV (Fig 2B). The response class prediction for the samples of the training set as responder (Prediction score >0) or as non-responder (Prediction score <0) gave correct categorization in 13/15 samples. In addition to sample T-16, sample T-03 was misclassified. Sample T-03 (non-responder) had a rare EGFR exon 20 9 BP insertion.

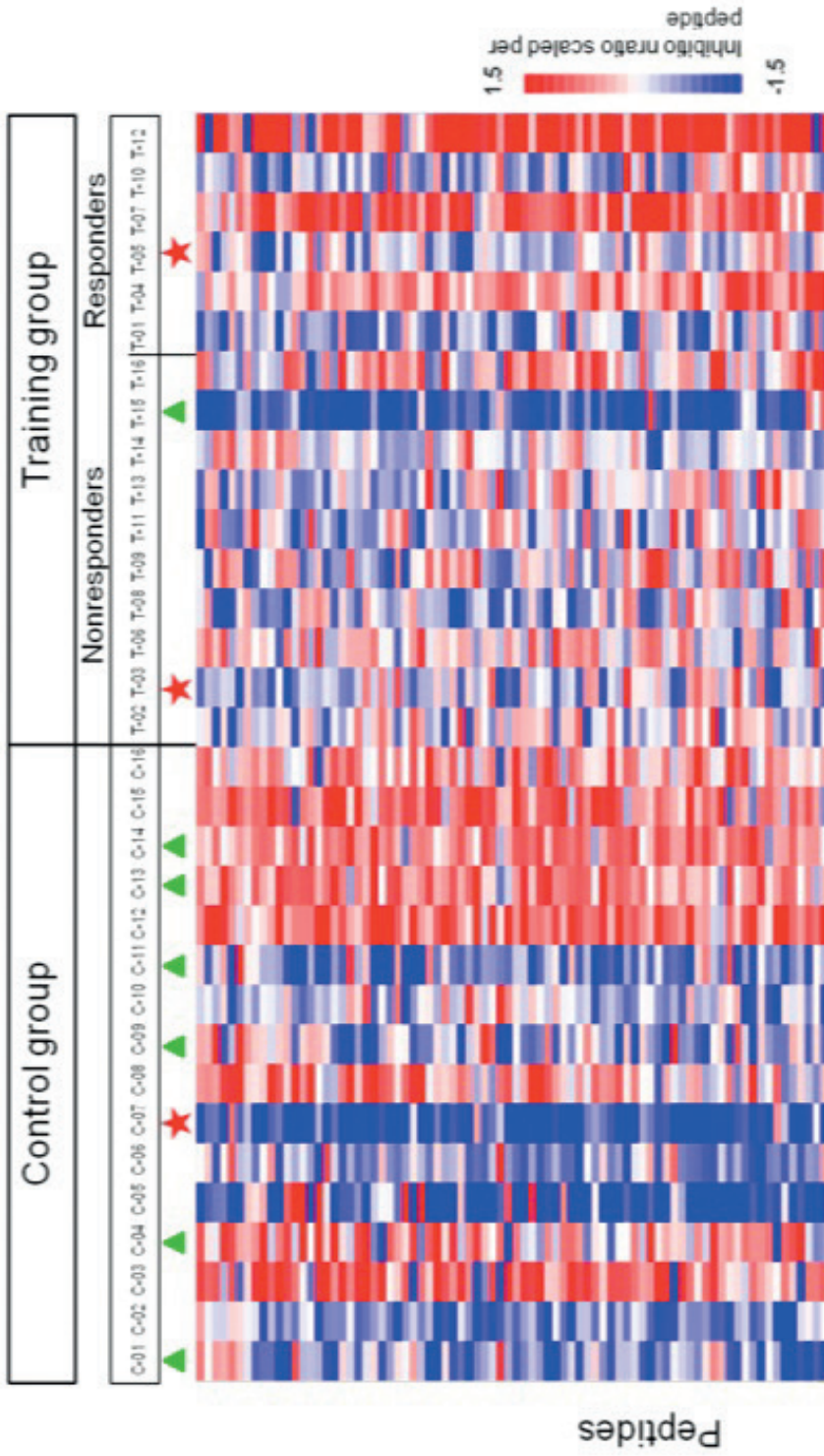
The error rate from this procedure was 15% (2 samples misclassified). From a set of 700 repeats of the cross validation procedure, but with the responder and non-responder labels randomly re-assigned to the samples (permutation test) the probability of obtaining such an error rate result by chance is $p<0.02$.

Application of the model to the blinded validation set

The prediction model built with the training set was applied to the inhibition ratio profiles. The inhibition profiles of the blinded validation set and their prediction scores are shown in Figure 3A and 3B. Subsequent comparison of this classification to the clinical response assessment revealed that of the 15 samples, 12 were classified correctly. V-01 (complete responder, adenocarcinoma with EGFR exon 19 mutation), V-15 (breast cancer metastasis) and V-14 (concomitant tuberculosis) were classified incorrectly. Histological analysis revealed a very low percentage (3%) of remaining tumor cells in sample V-01.

Mutation status and response prediction





Supplementary figure S2. Inhibition profiles of the training set and the control set. Inhibition ratios have been scaled per peptide. KRAS mutants are indicated by diamonds, EGFR mutants by a star.

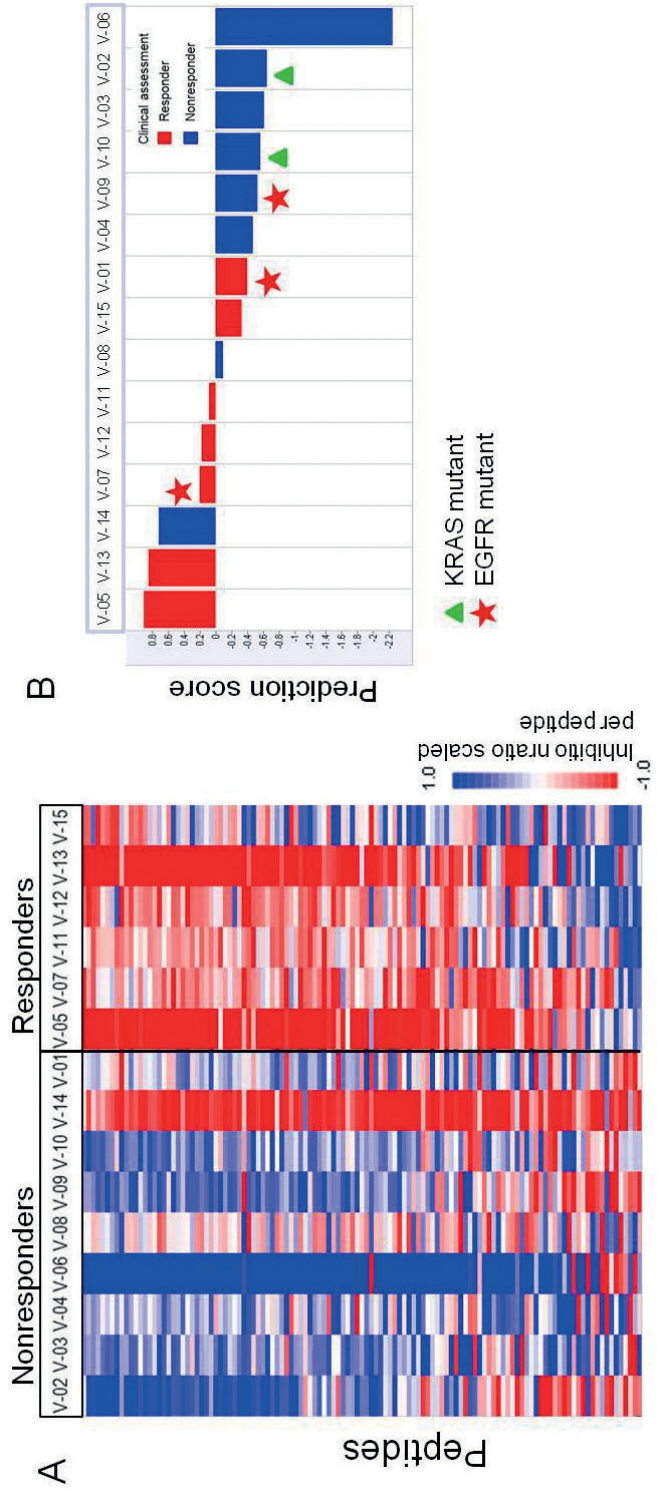


Figure 3A. Inhibition profiles as in Figure 2, for the validation set (n=15).

B. Response predicted by Leave One Out Cross Validation. Samples are ranked by predicted response and colored according to clinical assessment of response. KRAS mutants are indicated by diamonds, EGFR mutants by a star.

In the treated patient groups a total of 5 patients showed a EGFR mutation and 5 a KRAS mutation (Table 1). All except one patient (V-01) with an EGFR mutation were classified as responders. One patient (sample T03), with a rare EGFR exon 20 9 BP deletion, was classified as responder but was clinically a non-responder. All patients with KRAS mutation were correctly classified as non-responders (Figure 2B and 3B).

DISCUSSION

This study illustrates the potential of kinase activity profiling in tumor tissue of individual patients with NSCLC for predicting treatment outcome with erlotinib. A classifier was built based on inhibition profiles in a training set with known clinical response data. This classifier, as used in a blinded validation, correctly indicated likely response or non-response in 12 out of 15 patient samples. Only a few microgram of fresh (frozen) tissue is needed for the measurement of kinase activity with this assay.

A limitation of the study was that no paired pre-treatment and post-treatment samples were available. To test whether the treatment resulted in profiles differences compared to untreated tumor tissue (the situation in which a predictive test would be used in clinical practice), we compared the data from the training set to a matched group of untreated patients. Although results in some tumors (like near-complete responders T-05 and V-01) may be affected by treatment, the spectrum of inhibition profiles of treated tumors was homogeneous and comparable to that of untreated tumors. The feasibility study showed that it was possible to distinguish responders from non-responders after neoadjuvant erlotinib treatment.

Building a classifier for the training set was performed twice in separate experiments with different experimental conditions. In both cases, the prediction model correctly classified 13 out of 15 samples during LOOCV, which is more than what would be expected to occur by chance. Application of the classification model to a blinded validation set result in correct prediction of response for 12/15 samples. Patients with straight forward NSCLC were all diagnosed correctly except one patient who had a near complete response with very little viable tumor tissue left. The other two patients with misclassified results were patients with pulmonary comorbidity besides NSCLC, which may have influenced the kinase expression profile.

This one-step mix and measure test is performed directly on tissue lysate. Since enzyme activity measurements result in intrinsic signal amplification, no additional amplification step is needed. Overall differences in signal intensity could be attributed to differences in tissue input in the lysis batch and were eliminated by a batch wise normalization. Optimization of the sample input and spiked-in inhibitor concentration improved the separation between responders and non-responders. The multiplex assay set-up allows for processing of a large number of samples simultaneously and allows the analysis of sufficient number of replicates for statistical analysis of the data.

Four samples with EGFR exon 19 mutations were present among classified responders, in concordance with expectations. One sample that had a rare EGFR exon 20 9 BP deletion, did classify as responder by Pamindex but was clinically a non-responder. KRAS mutations (five) were only found in classified non-responders, also as expected. Hence, for those patients with activating EGFR or with KRAS mutation response prediction using kinase activity profiles was consistent with that based on the mutation status of the analysed tumor. However, in the present study 12 patients were indicated as a responder, 5 of which harboured an EGFR mutation. In the current clinical practice, based on mutation analysis, only these last 5 would have received treatment with erlotinib. Based on the predictive assay described here, 7 more patients would be eligible for erlotinib treatment, with one of those being a (clinical) non-responder. To establish the robustness of this functional inhibition classifier for response to erlotinib treatment, further validation is necessary in pre-treatment samples and comparison with treatment results. However, the present study shows promising and results for the use of kinase activity profiling for response prediction, in a 2 hour assay. For future optimization and validation with strict inclusion criteria and pretreatment samples should be used to improve accuracy of the test.

CONCLUSION

Kinase activity profiling may be a promising step towards personalized medicine for lung cancer patients. For the future functional testing using kinase activity profiling in the absence and presence of targeted agents such as erlotinib should be further validated in prospective clinical trials.

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