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Predictive signature of response to EGFR tyrosine kinase inhibitors in non-small-cell lung cancer cells by tyrosine kinase activity profiling

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Abstract

Background: There is an urgent need to develop companion diagnostics for response prediction to increase the success rate of anticancer drug development. Constitutively activated protein kinases are major targets for cancer treatment[1-3]. However, blockade of target molecules may not necessarily lead to treatment response. Thus, we attempted to investigate the consequences of target kinase inhibition by comprehensive tyrosine kinases activity profiling[4-6], which can be applied to establish a response prediction signature. Here, we employed clinically validated EGFR-targeted inhibition as in vitro model utilizing PamChip® peptide micro-arrays.

Methods: Six lung cancer cell lines harboring either wild-type EGFR (A549 and H441) or mutant EGFR (HCC4006, PC9, HCC827, and H1650) were used. Chemosensitivity to EGFR inhibitors (gefitinib, erlotinib, and afatinib) was evaluated by MTT assay and IC50 values were obtained. For assessment of protein tyrosine kinase activity, cell lysates were prepared, aliquoted and stored at -80 degree until use. The tyrosine kinase activity of the lysates was measured in real time with or without EGFR TKIs on PamChip® peptide micro-arrays, containing 144 peptides derived from known human phosphorylation sites. Data analysis was carried out on both basal and inhibition profiles. Inhibition profiles were calculated for each EGFR TKI by taking the $\log_2$-ratio of peptide phosphorylation measured with or without EGFR. Peptides that did not show increase of signal in time (indicative of kinase activity) were excluded from the analysis.

Results: Basal tyrosine kinase activity profiles showed a clear segregation between EGFR driven (HCC4006, PC9, and HCC827) and EGFR independent (H1650, H441 and A549) cells. Additionally, overall tyrosine kinase activities in EGFR mutant cells were higher than those in EGFR wild-type cells. Based on IC50 values for three EGFR TKIs, the cell lines were divided into responders (PC9, HCC4006 and HCC827 with IC50 values ≦0.1 μmol/L), non-responders (H1650, H441 and A549 with IC50 values >1 μmol/L). Basal phosphorylation signals on a number of peptides differed between responders and non-responders. The responders showed phosphorylation on sites responsible for activating RAF, ERK1/2 and MAPK7 and on sites for inactivating the phosphatases PP2AB and PTN11. The phosphorylation was absent in the non-responders, possibly as a consequence of constitutive activation of the RAS/RAF pathway. This peptide set was suggested to serve as a predictive signature of response to EGFR TKIs.

Conclusions: Kinase activity profiles reflect the biochemical consequences of EGFR mutant non-small-cell lung cancer. Tyrosine kinase activity profiling may serve as a companion diagnostic in drug development and this should be further explored.

Methods

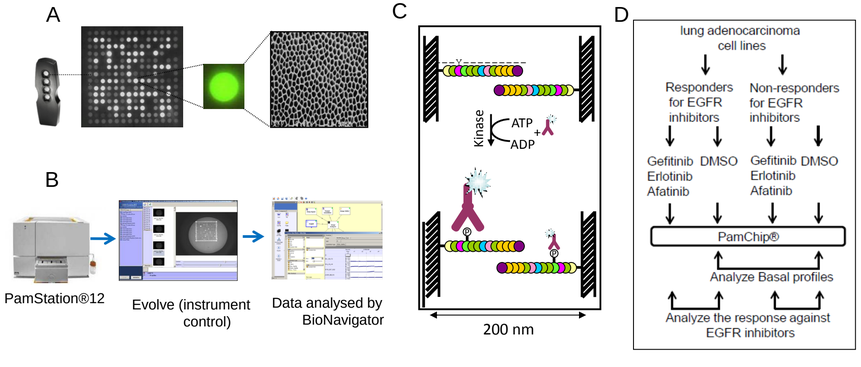

Figure 1. (A)(B) PamStation ® 12 instrument (Pamegene International BV, 's-Hertogenbosch, Netherland) and PamChip® arrays used for kinase activity profiling[4-6]. (C) The reaction takes place on peptide spots that are covalently bound to the array. Peptide phosphorylation through active kinases can be monitored in lysates of cell lines by real time fluorescent readout using a fluorescently labeled anti-phospho-tyrosine antibody. (D) The flow of examination.

Results

Table 1. Cell line properties and growth-inhibitory effect of EGFR inhibitors(gefitinib, erlotinib, afatinib).

Cell line	Histology	EGFR status	KRAS status	PTEN status	ERBB2 status	Gefitinib IC50 (μM) ±SD	Erlotinib IC50 (μM) ±SD	Afatinib IC50 (μM) ±SD
A549	Adenocarcinoma	Wild Type	G12S	Wild	Wild	16.08±1.06	13.28±7.80	2.08±0.21
NCI-H1650	Adenocarcinoma	E746_A750del	Wild	Del	Wild	47.95±0.13	12.37±4.17	3.49±0.71
NCI-H441	Papillary adenocarcinoma	Wild Type	G12V	Wild	Wild	19.09±2.21	3.31±1.18	3.56±0.23
HCC4006	Adenocarcinoma	L747_E749del	Wild	Wild	Wild	0.03±0.001	0.03±0.003	0.002±0.00006
HCC827	Adenocarcinoma	p.E746_A750del	Wild	Wild	Wild	0.03±0.002	0.03±0.004	0.002±0.0001
PC-9	Adenocarcinoma	p.E746_A750del	Wild	Wild	Wild	0.03±0.005	0.03±0.004	0.0023±0.0001

Non-responders to EGFR inihibitors Responders to EGFR inihibitors

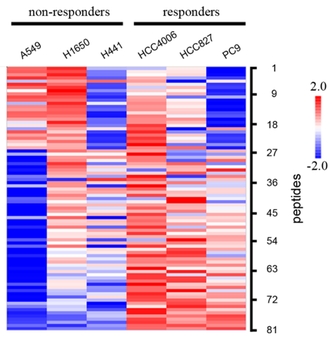

Figure 2. Peptide scaled heatmap of tyrosine kinase activity in the absence of inhibitors. Heatmap is represented by $\log_2$ signal intensity. Of the 144 peptide substrates on the PamChip®, 81 peptides show an increase in signal in time and are included in the analysis.

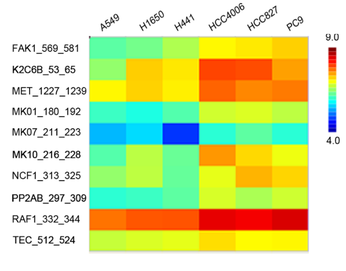

Figure 4. The signal intensity and identity of 10 peptides with significant difference (p-value ≤ 0.05) between responders and non-responder on basal profiling.

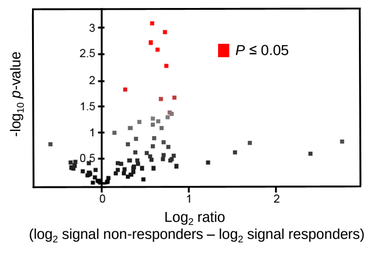

Figure 3. Volcano plot analysis on basal profiling between responders and non-responders to EGFR inhibitors. 10 peptides have significant difference (p-value ≤ 0.05) between responders and non-responders.

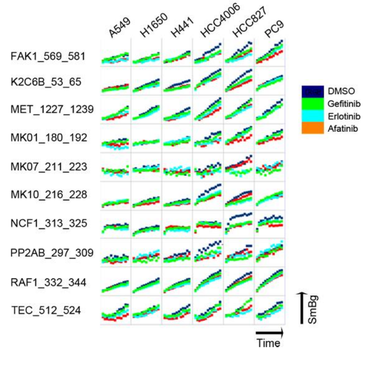

Figure 5. The time course of phosphorylation for 10 peptides in figure 4.

Table 2. Characteristics of 10 peptides.

No.	Peptide	Sequence	Phosphorylation position	Potential upstream kinases (phospho-ELM)	Swiss-Prot accession
1	FAK1_569_581	RYMEDSTYYKASK	[570, 576, 577]	Src, INSR, IGF1R, FAK, Fgr, Met	P06213
2	K2C6B_53_65	GAGFGSRSLYGLG	[62]	-	P04529
3	MET_1227_1239	RDMYDKEYYSVHN	[1230, 1234, 1235]	Met, Ron	P08581
4	MK01_180_192	HTGFLTEYVATRW	[187]	EPHA3, EPHA4, EPHA7, JAK2, MAP2K1, MEK1	P28482
5	MK07_211_223	AEHQYFMTEYVAT	[215, 220]	-	Q13164
6	MK10_216_228	TSFMMTPYVVTRY	[223, 228]	MKK4, MAP2K3, MAP2K4, MAP2K6, MAP2K6_MAP2K3_MAP2K4_MAP2K7_group	P53779
7	NCF1_313_325	QRSRKRLSQDAYR	[324]	-	P14598
8	PP2AB_297_309	EPHVTRRTPDYFL	[307]	-	P62714
9	RAF1_332_344	PRGQRDSSYYWEI	[340, 341]	SRC, JAK2, INSR, IGF1R, EPHA3, EPHA4, EPHA7	P04049
10	TEC_512_524	RYFLDDQYTSSSG	[513, 519]	TEC, JAK2	P42680

Conclusions

- The phosphorylation activity in responders tended to be higher than that of non-responders on basal profiling Heatmap. The percentage of inhibition by gefitinib, erlotinib, or afatinib present in the assay mix was not significantly different between the responders and non-responders.
- Of 81 peptides that showed an increase in signal in time, the phosphorylation activities on 10 peptides were significantly higher for responders than for non-responders and showed inhibition by EGFR inhibitors.
- These peptide are associated with EGFR pathway[7-11]. Thus, PamChip® demonstrated that the EGFR pathway was important for lung adenocarcinoma cell lines with a good response against EGFR inhibitors.
- This profiling system may be utilized to detect the important pathways for new molecular targeted agents.
- Kinase activity measurement on PamChip® should be further explored to predict the response of human tissue to EGFR inhibitors and for other cancer cell lines to determine the response to other tyrosine kinase inhibitors.

References

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