Downstream EGFR Protein Phosphorylation and Gefitinib Inhibition in Non-Small Cell Lung Cancer Cells Detected With Multiplex Phosphoprotein Assays

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Introduction

The purpose of this study was to detect the effects of gefitinib (marketed as Iressa by AstraZeneca Pharmaceuticals) on the phosphorylation of downstream targets of EGFR (Figure 1) and to show the application of Bio-Plex® phosphoprotein assays to drug discovery based on signal transduction pathways. Gefitinib, an inhibitor of epidermal growth factor receptor (EGFR), is used to treat non-small cell lung cancer (NSCLC). The response of NSCLC to gefitinib is related to mutations occurring within the EGFR kinase domain. Because Bio-Plex phosphoprotein assays (xMAP technology) can detect multiple phosphoprotein targets from a single cell lysate sample, they are a useful tool to reveal the phosphorylation state of targeted proteins along their signal transduction pathways. We used Bio-Plex phosphoprotein assays to probe the phosphorylation state of three NSCLC cell lines treated by gefitinib followed by epidermal growth factor (EGF) stimulation (Figure 2). The NSCLC cell lines we used include a wild-type (H-1734) and two mutated strains (H-1650 and H-1975). H-1650 contains a deletion mutation (del L747-P753) and responds to gefitinib. H-1975 has the double point mutations L858R and T790M. The cellular response to gefitinib caused by the L858R mutation is reversed by the T790M mutation. The six EGFR downstream targets in this study are phosphorylated: p-Akt, p-MEK1, p-ERK1/2, p-GSK-3α/β, p-p70 S6K, and p-p90RSK. Results of the multiplex phosphoprotein assays were compared with individual western blot results (Figures 3 and 4).

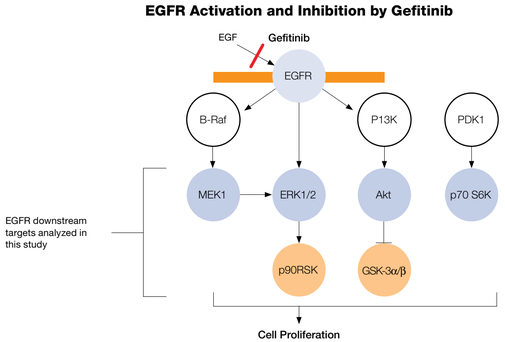

Fig. 1. Schematic of the signal transduction pathway downstream of EGFR. We used Bio-Plex phosphoprotein assays to detect the six phosphoprotein targets highlighted at bottom. The drug gefitinib inhibits EGFR stimulation.

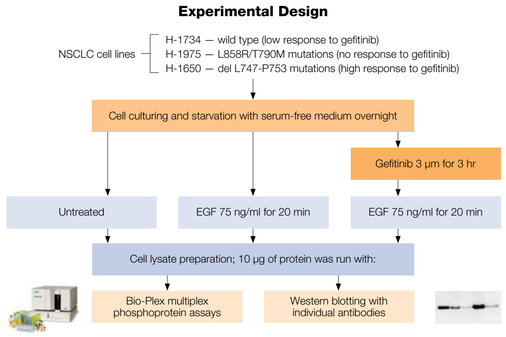

Fig. 2. Protocol to detect the six phosphoprotein targets downstream of EGFR that are shown in Figure 1. Experiments were performed on three NSCLC lines using both the Bio-Plex phosphoprotein assay and western blot analysis.

Results

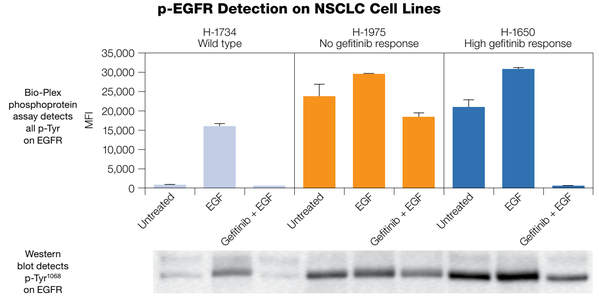

Fig. 3. The Bio-Plex assay compared well with western blot band intensity in detection of phosphorylated EGFR. Gefitinib inhibits the EGFR pathway but its effect varies among three cell cultures (H-1734, H-1975, H-1650). MFI = mean fluorescence intensity. Error bars indicate standard deviations.

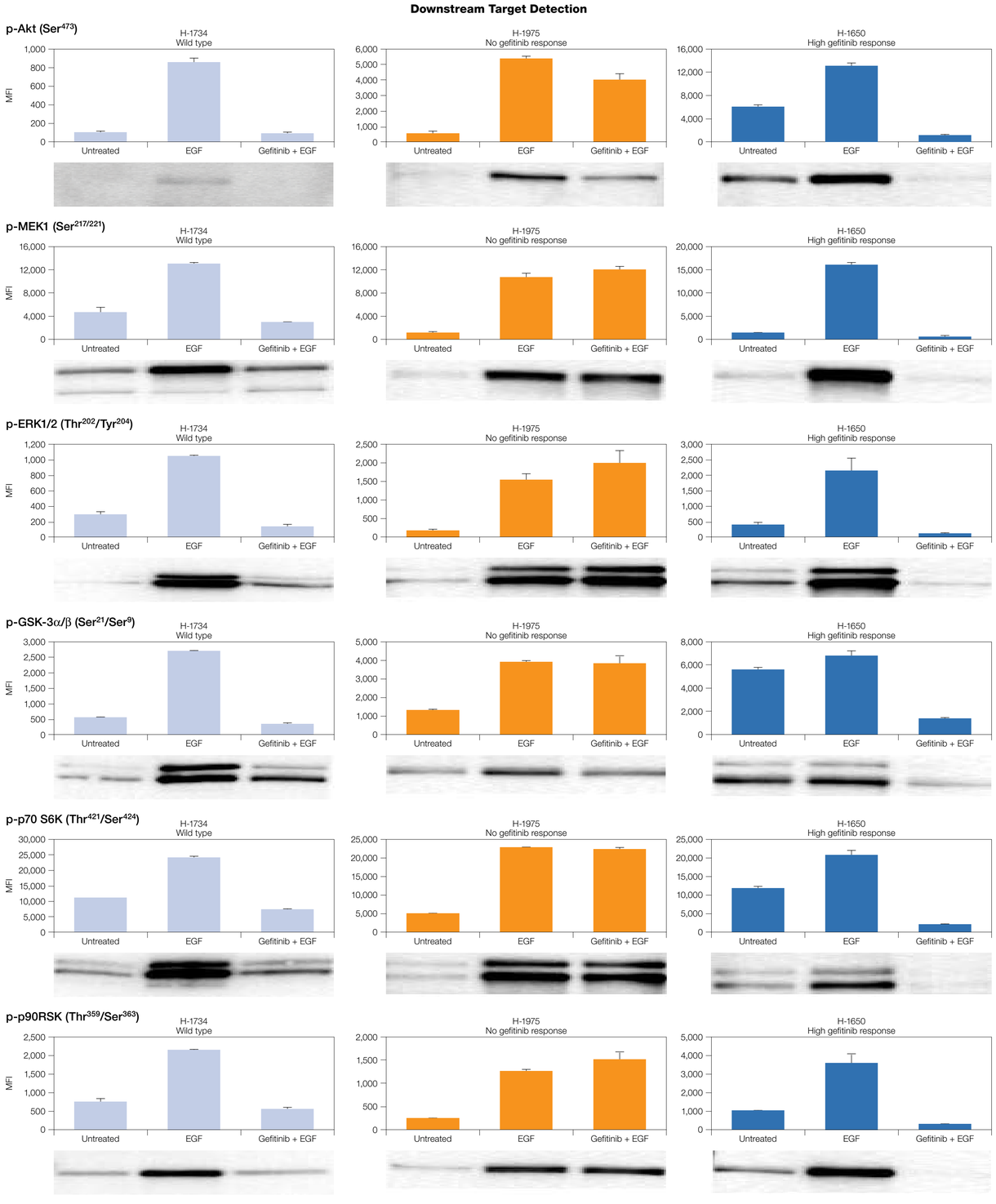

Fig. 4. The Bio-Plex assay compared well with western blot in detecting six phosphoprotein targets downstream of EGFR. Gefitinib inhibits the EGFR pathway but its effect varies among three cell cultures (H-1734, H-1975, H-1650).

Table 1. Magnitude of gefitinib inhibitory effect on six phosphoprotein targets in three NSCLC cell lines. Numbers represent the fold decline in phosphorylation measured between EGF and EGF + gefitinib lysates, calculated using MFI from the Bio-Plex phosphoprotein assay.

NSCLC Cell Line	p-Akt	p-MEK1	p-ERK1/2	p-GSK-3α/β	p-p70 S6K	p-p90RSK
H-1734	8.0	4.3	5.6	6.3	3.3	4.0
H-1975	1.4	0.9	0.8	1.0	1.0	0.8
H-1650	15.0	45.0	20.4	4.7	11.5	18.9

Conclusions

We measured tyrosine phosphorylation of EGFR and six downstream phosphoproteins in three NSCLC cell lines with and without gefitinib treatment. For all tested cell lysates, the MFI of the Bio-Plex assay correlated very well with the band intensity on western blot. The multiplex assay needed only 10 µg of cell lysate protein for each sample. Western blot analysis needed 60 µg of protein per sample because each target was probed with individual anti-phospho-specific antibody.

Among three NSCLC cell lines, the wild type (H-1734) showed moderate inhibition of phosphorylation on the downstream targets by gefitinib. The cell line H-1650, mutated by a deletion from L747 to P753, showed dramatic phosphorylation inhibition on all tested targets. The Bio-Plex assay measured signal decreases between EGF- and gefitinib/EGF-treated samples from 4.7-fold to 45-fold (Table 1). However, cell line H-1975, which carried the double point mutation (L858R and T790M), did not respond to the drug with the exception of p-Akt, which showed a slight signal decrease between EGF- and gefitinib/EGF-treated samples (1.4-fold). No other targets showed inhibition by gefitinib. These results correspond with studies showing that the second point mutation (T790M) reverses the drug response because of the substitution of methionine for threonine at position 790, resulting in steric hindrance of the drug binding to EGFR.

Bio-Plex phosphoprotein assays are useful tools in studying signal transduction pathways and revealing the phosphorylation status of multiple targets in anticancer drug discovery.

References

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07-0161 0207 Rev A