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tech note 5579

Multiplex Phosphoprotein Assays: Detection of Downstream Epidermal Growth Factor Receptor Protein Phosphorylation and Gefitinib Inhibition in Non-Small Cell Lung Cancer Cells

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Introduction

The purpose of this study was to detect the effects of gefitinib (marketed as Iressa by AstraZeneca International) on the phosphorylation of downstream targets of epidermal growth factor receptor (EGFR; Figure 1), and to show the application of Bio-Plex phosphoprotein assays in drug discovery based on signal transduction pathways.

The EGFR tyrosine kinase plays an important role in regulating essential cellular functions such as cell proliferation, survival, and differentiation. It has been an important targeted protein for anticancer drug discovery because inhibition of EGFR may suppress tumor growth. This anticancer effect is mediated by inhibition of phosphorylation of EGFR induced by growth factors, such as epidermal growth factor (EGF).

Gefitinib, an inhibitor of 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 (Sordella et al. 2004). Because Bio-Plex phosphoprotein assays (based on xMAP technology) can detect multiple phosphoprotein targets from a single cell-lysate sample, they are useful tools for revealing the phosphorylation status of the targeted protein along its signal transduction pathways.

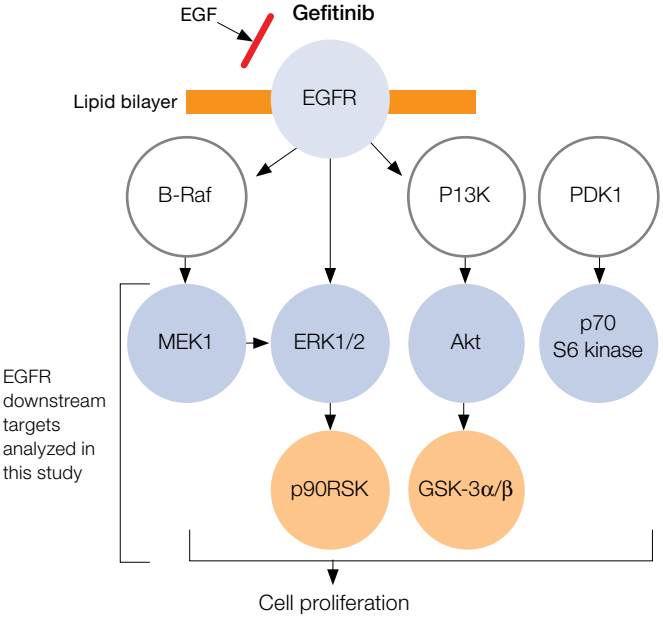

Fig. 1. Schematic of the signal transduction pathway downstream of EGFR. We used Bio-Plex phosphoprotein assays to detect the six phosphoprotein targets highlighted at bottom as well as p-EGFR. The drug gefitinib inhibits EGFR stimulation.

We used Bio-Plex phosphoprotein assays to probe the phosphorylation status of three NSCLC cell lines treated by gefitinib followed by EGF stimulation. We compared the results with western blotting results. The NSCLC cell lines we used included a wild-type strain (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. We focused on six EGFR downstream targets: p-Akt, p-MEK1, p-ERK1/2, p-GSK-3α/β, p-p70 S6 kinase, and p-p90RSK.

Methods

Cell Culture and Cell Lysate Preparation

The three NSCLC cell lines were obtained from American Type Culture Collection (ATCC). All cell lines were cultured in RPMI-1640 with 10% fetal bovine serum. When cells reached about 90% confluence, we changed the culture medium to RPMI-1640 without serum and incubated the cells at 37°C overnight. Three conditions were applied to each cell line: untreated, EGF-stimulated, and gefitinib-treated/EGF-stimulated. Gefitinib was added into the serum-free medium at a final concentration of 3 µM and incubated for 3 hr, followed by EGF stimulation at 75 ng/ml for 20 min. Cells were washed and lysed with a Bio-Plex cell lysis kit according to the instructions. Total cell lysate protein concentration was measured by a Bio-Rad *DC*™ protein assay and the lysates were stored at –20°C for further analysis.

Bio-Plex Phosphoprotein Assays

The Bio-Plex p-EGFR assay was run as a singleplex assay. The assay cannot be multiplexed with other phosphoassays because it detects phosphorylated tyrosine, which is present in all other phosphoassays. To detect downstream EGFR targets, we ran a multiplex phosphoprotein assay for the six targeted

phosphoproteins mentioned above. The Bio-Plex assays used 10 µg of total lysate protein for each well. Samples were tested in duplicate following the assay kit instructions. Reported results include median fluorescence intensity (MFI), standard deviation of mean MFI, and percent coefficient of variation (%CV).

Western Blot Analysis

For comparison to the Bio-Plex results, 10 µg of total lysate protein for each sample was loaded onto a Criterion™ XT Bis-Tris 4–12% gel. After transfer to a membrane, targeted protein was probed with corresponding antiphospho-epitope-specific antibodies (Cell Signaling Technology, Inc.) at room temperature overnight followed by an HRP-conjugated secondary antibody. Images were developed with chemiluminescent substrate (SuperSignal west femto substrate, Pierce Biotechnology, Inc.) and analyzed using a Molecular Imager® Gel Doc™ imaging system.

Results and Discussion

We measured tyrosine phosphorylation of EGFR and six downstream phosphoproteins in three NSCLC cell lines with and without gefitinib treatment. For all tested cell lysate samples, the MFI of Bio-Plex assays correlated very well with band intensity on western blots.

Hypothesized EGFR-Gefitinib Interactions

Gefitinib inhibits EGFR by blocking EGF from binding to it. About 10% of NSCLC patients are responsive to gefitinib and almost all responsive tumors harbor EGFR mutations, but it is not clear which downstream proteins are affected (that is, are not phosphorylated) due to the effects of gefitinib on EGFR (Figure 1). Some research suggests that phosphorylation of downstream targets Akt, ERK, and STAT5 are affected (Sordella et al. 2004). We investigated some of these downstream proteins by using specific cell lines that carried EGFR mutations around several phosphorylated tyrosine motifs.

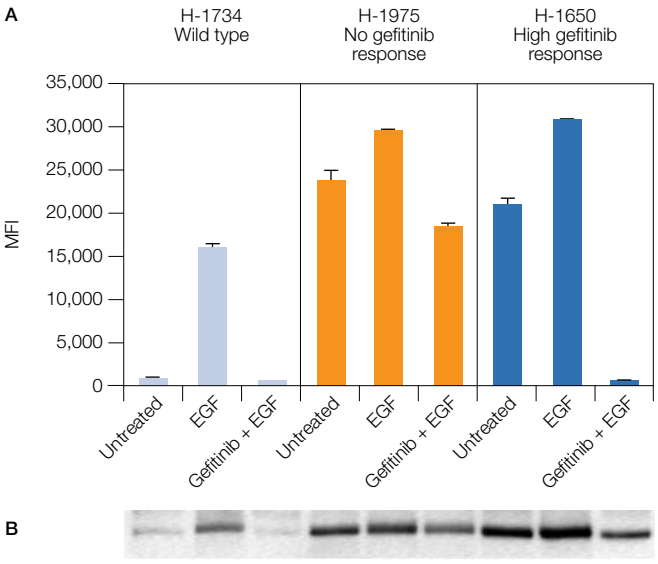

Fig. 2. P-EGFR detection on non-small cell lung cancer cell lines.
A, the Bio-Plex phosphoprotein assay detects all p-Tyr on EGFR; **B**, western blotting detects p-Tyr^{1068} on EGFR. The Bio-Plex assay compared well with western blot band intensity in detection of p-EGFR with one exception (rightmost column). Gefitinib inhibits the EGFR pathway but its effect varies among three cell cultures. MFI, median fluorescence intensity from duplicate samples. Error bars indicate standard deviations of mean MFIs.

Phospho-EGFR Detection

The Bio-Plex p-EGFR assay detected dramatic phosphorylation decreases in H-1734 and H-1650 cell lines after gefitinib treatment (Figure 2). Cell line H-1975, with a double point mutation, did not show an obvious phosphorylation decrease. Compared with the Bio-Plex data, western blotting demonstrated gefitinib inhibition on EGFR phosphorylation with H-1734 cells but much less inhibition with H-1650 cells. This discrepancy between the Bio-Plex assay and the western blot can be understood by considering the different antiphospho antibodies used in the two procedures. The Bio-Plex assay used a generalized p-tyrosine antibody, whereas the western blot used an antibody specific to the p-tyrosine-1068 epitope. EGFR contains multiple p-tyrosine sites, so the Bio-Plex assay detected overall decreases in tyrosine phosphorylation, whereas western blotting detected decreases in phosphorylation of tyrosine in one epitope only.

EGFR Downstream Phosphoprotein Detection

The results for six EGFR downstream targeted proteins are presented in Figure 3.

Six downstream targets (p-Akt, p-MEK1, p-ERK1/2, p-GSK-3α/β, p-p70 S6 kinase, and p-p90RSK) were tested with the multiplex phosphoprotein assay using 20 µg of total cell lysate protein for duplicate data. Western blotting results required 60 µg of total cell lysate protein per sample and generated only one data point. Good correlation was demonstrated between the Bio-Plex assays and western blots in all tested samples and targeted proteins. Gefitinib inhibited the phosphorylation of all six downstream targets in NSCLC cell lines H-1734 and H-1650. In general, the inhibition was stronger on H-1650 than on the wild-type cell line, H-1734. These results suggest that the inhibition of EGFR phosphorylation by gefitinib affects multiple downstream targets involved in different signal transduction pathways. Contrary to cell line H-1650 response, the double point-mutated cell line H-1975 showed resistance to gefitinib inhibition. We saw no decreased phosphorylation on five of six downstream targets (p-Akt showed a phosphorylation decrease of about 30% after gefitinib treatment). These results are consistent with findings that the secondary mutation T790M could be responsible for drug resistance (Kwak et al. 2005).

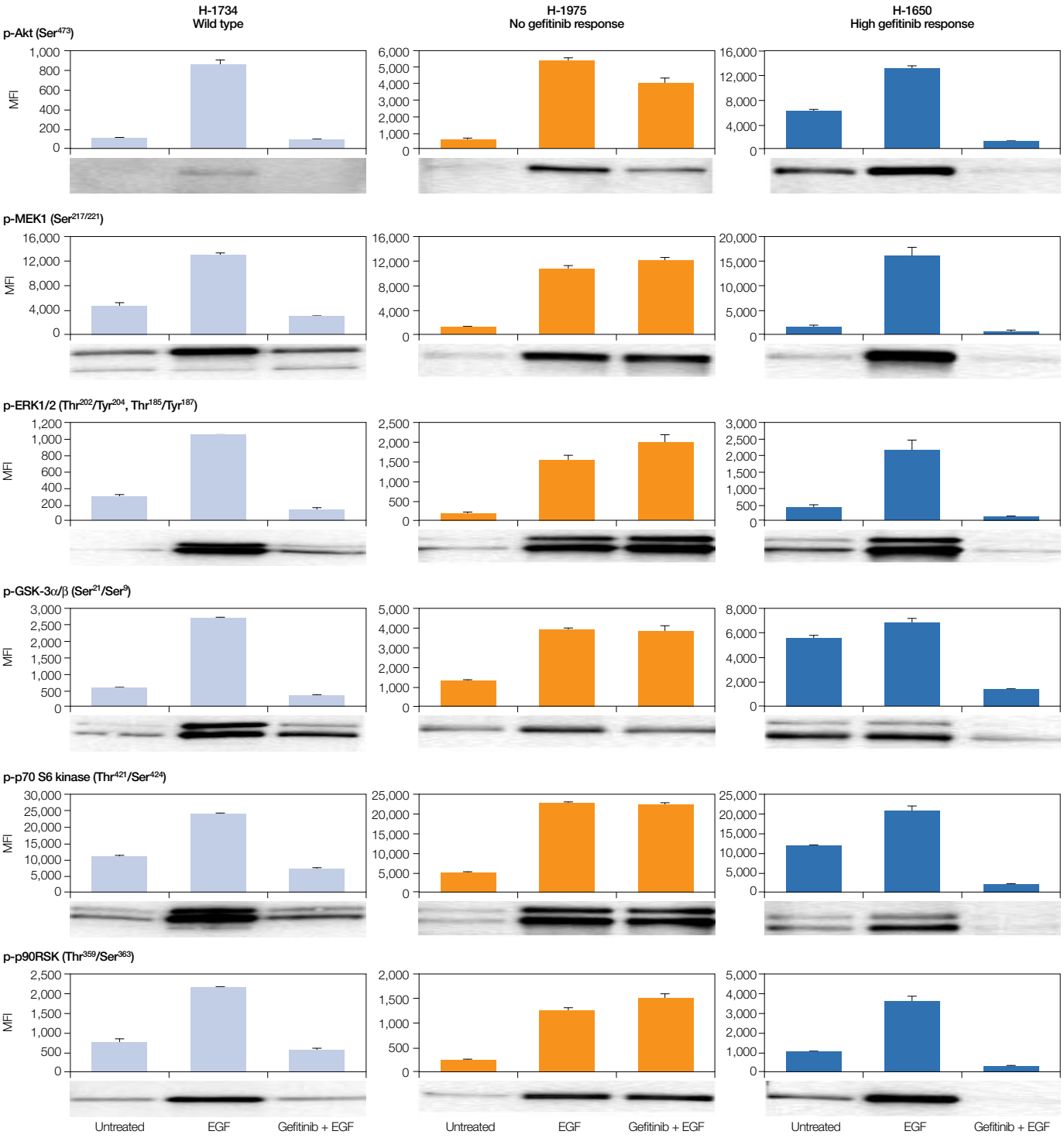

Fig. 3. Detection of EGFR downstream phosphoprotein targets. The Bio-Plex assay compared well with western blotting in detecting six phosphoprotein targets downstream of EGFR. Gefitinib inhibits the EGFR pathway but its effect varies among three cell cultures (H-1734, H-1975, H-1650). MFI, mean fluorescence intensity from duplicate samples. Error bars indicate standard deviations of mean MFIs.

Conclusion

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

Kwak EL et al., Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib, Proc Natl Acad Sci USA 102, 7665–7670 (2005)

Sordella R et al., Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways, Science 305, 1163–1167 (2004)

Information in this tech note was current as of the date of writing (2007) and not necessarily the date this version (rev A, 2007) was published.

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