Bio-Plex® suspension array system

tech note 5578

Comparison of Protein Phosphorylation in Cell Line and Xenograft Samples by Bio-Plex® Suspension Array and Western Blotting Techniques

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Introduction

Receptor tyrosine kinases (RTKs) mediate growth, differentiation, and developmental signals in cells by adding phosphate groups to substrate proteins to change the activation state of the proteins. When the genes encoding RTKs are altered or mutated, they can become potent oncogenes, causing the initiation and progression of a number of cancers. These pathways play a key role in the development of new drug therapies. Clinicians need to understand the degree of activation of a particular pathway and its engagement with downstream components in order to target a set of interconnected kinase-driven events along a signaling pathway. This will enable efficacious targeting of treatment.

In order to efficiently identify patients most likely to benefit from targeted drug therapy, it is essential to develop new laboratory techniques. Standard laboratory and clinical assays such as immunohistochemistry, enzyme-linked immunosorbent assays (ELISAs), and western blots can detect expression of only a limited number of proteins at once. In contrast, the Bio-Plex bead-based platform (based on Luminex technology) can detect cell-signaling events that involve up to 100 protein targets in a single sample. In a partnership, Cell Signaling Technology, Inc. (CST) and Bio-Rad Laboratories, Inc. developed, optimized, and validated Bio-Plex suspension array assays to detect and measure therapeutic targets and determinants of therapeutic efficacy.

The objective of this study was to perform Bio-Plex assays using in vitro and in vivo samples of the human non-small cell lung cancer line HCC827 (adenocarcinoma) and to compare the results to western blots.

Methods

Cell Lysate Preparation

HCC827 cells (American Type Culture Collection (ATCC)) were grown to 85% confluence and starved overnight. Cells were either untreated, treated with 100 ng/ml of epidermal growth factor (EGF, from CST), or inhibited with 1 mM gefitinib (marketed as Iressa by AstraZeneca International) for 2 hr and then treated with 100 ng/ml EGF. Cells were rinsed with phosphate-buffered saline (PBS), then lysed with Bio-Plex cell lysis buffer, sonicated three times (20 sec pulses), and centrifuged at 3,300 rpm for 10 min to remove cell debris. Supernatants were collected and the protein concentration in each sample was measured using the Bio-Rad *DC*™ protein assay.

Xenograft Lysate Preparation

We injected 1 x 10^7 HCC827 cells subcutaneously into each of ten 8-week-old female mice (Taconic Farms, Inc.). When the tumors were 1 cm^3, half the mice were administered vehicle control (100 µl Tween 80), and the remaining mice were given 150 mg/kg of gefitinib dissolved in Tween 80 by oral gavage. Tumors were harvested 24 hr after treatment. Tissue (30 mg) was excised from each tumor, placed in Bio-Plex cell lysis buffer, and homogenized by mechanical lysis. The samples were centrifuged to remove debris, and the protein concentrations were measured using the Bio-Rad *DC* protein assay.

Bio-Plex Assays

Targets known to be affected by gefitinib treatment were selected for further analysis using the Bio-Plex system. The lysates (0.2 mg/ml of sample assayed in duplicate) were evaluated for total and phosphorylated ERK1/2, epidermal growth factor receptor (EGFR), and S6 ribosomal protein according to the Bio-Plex assay instructions.

Western Blotting

The lysates were evaluated for total and phosphorylated ERK1/2, EGFR, and S6 ribosomal protein using CST's total and phospho-specific antibodies. Protein (18 µg) was loaded in each well and run on a 4–20% Tris-glycine gradient gel (Jule, Inc.) using the western blot assay protocol recommended by CST (http://www.cellsignal.com/support/protocols/Western_Milk.jsp). Proteins were transferred onto nitrocellulose membrane and blocked for 1 hr with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.1% Tween 20 (TBST). Primary antibodies (CST) were diluted 1:1,000 in blocking buffer and the blots were incubated overnight at 4°C. Blots were washed three times with TBST. Goat anti-rabbit HRP-linked antibody was diluted at 1:2,000 in TBST + 5% nonfat dry milk (w/v, CST). Blots were incubated for 1 hr at room temperature, then washed three times in TBST. Blots were developed with LumiGLO and peroxidase reagents (CST). Western blots were scanned (Epson Perfection 1240 U scanner), and quantitated with Scion densitometry software (Scion Corporation).

Statistical Comparison of Bio-Plex Assays and Western Blots

We used the assay results from the nude mouse experiment to determine whether the Bio-Plex assay could detect phosphoproteins in tumor tissues. We used Student's *t*-test to statistically compare treated and control mice (considered different when $p < 0.05$). We then used Spearman's correlation test to compare the Bio-Plex assay results with western blot results obtained by Scion densitometry software.

Comparison of In Vitro and In Vivo Results With Phosphorylation Index (PI)

For comparison, we calculated a PI for each analyte in the in vitro and in vivo experiments. For each target protein, PI = (phosphoprotein MFI/total protein MFI) x 100, where MFI is the median fluorescence intensity recorded in each Bio-Plex assay. The PI normalizes the level of phosphorylation based on the total amount of protein for each analyte. The PI for each analyte was compared by determining the fold decrease in PI between the cell lysates treated with EGF or EGF + gefitinib and the control and gefitinib-treated mice.

Results and Discussion

Comparison of Bio-Plex and Western Blots Using HCC827 Cell Lysate

In a side-by-side comparison, the Bio-Plex assay and western blots showed equivalent detection of total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein (Figure 1). Both techniques showed marked reduction of phosphorylated ERK1/2, EGFR, and S6 ribosomal protein in the cell lysate from gefitinib-treated HCC827 cells compared to EGF-treated cells. Similar patterns of phosphorylation were detected by the Bio-Plex assay and the western blot, as imaged by Scion software (Figure 1).

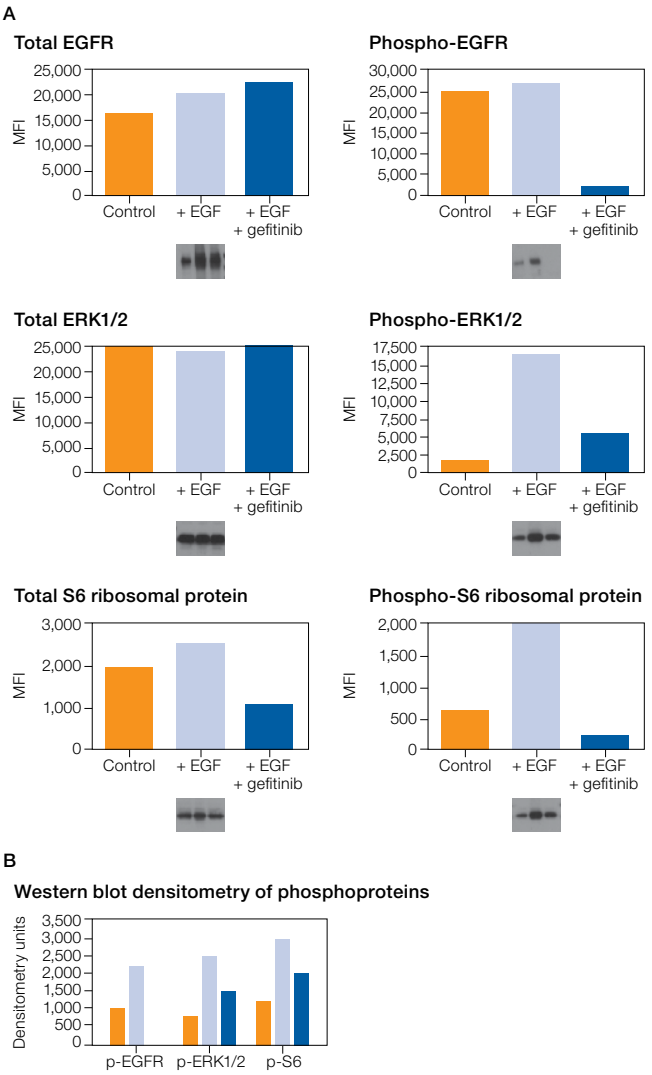

Fig. 1. Inhibited phosphorylation of three target proteins by gefitinib in HCC827 cell lysate treated with EGF. A, graphs indicate Bio-Plex assay results; images are western blots; **B**, western blots quantitated by densitometry yielded similar phosphorylation patterns to Bio-Plex assay results. The densitometry reading for p-EGFR, + EGF + gefitinib was 0. ■, control; ■, + EGF; ■, + EGF + gefitinib.

Ability of Bio-Plex Assays to Evaluate Phosphoproteins in Solid Tumors

The Bio-Plex assays and western blots detected total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein equivalently. The Bio-Plex assays detected decreased phosphorylation of the three target proteins in mouse tumors treated with gefitinib vs. untreated controls (results using Student's *t*-test, with $n = 5$, were all $p < 0.05$; Figure 2). Densitometer-quantitated western blot results for the same comparisons are also shown in Figure 2. There was a significant correlation between the results obtained by the Bio-Plex assay and those obtained by western blotting (results using Spearman's correlation test, with $n = 30$, were $r = 0.5087$ and $p = 0.004$), confirming the two techniques perform equivalently.

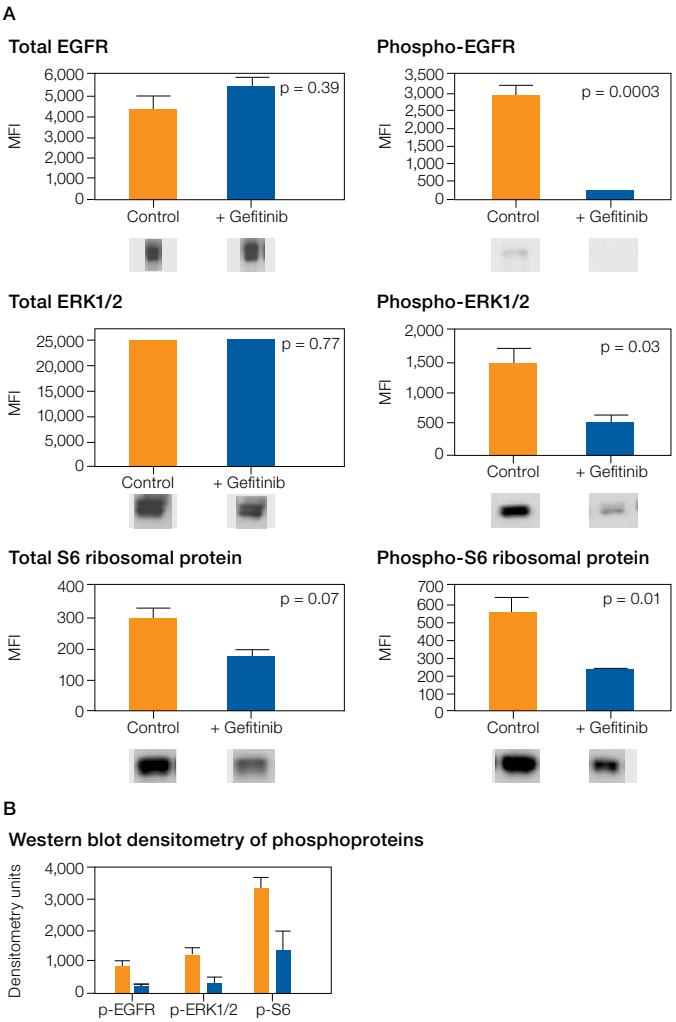

Fig. 2. Inhibited phosphorylation of three target proteins by gefitinib in HCC827 xenograft lysate from mouse tumors. A, means of median fluorescence intensity (MFI) ± the standard error of the mean (SEM) for five treated mice compared with five controls. Graphs indicate Bio-Plex assay results; images are western blots; **B**, western blots quantitated by densitometry showed the same patterns as (and correlated significantly with) Bio-Plex assay results. ■, control; ■, + gefitinib.

Phosphorylation Ratios of HCC827 Cell and Xenograft Lysates

Comparison of PI values confirmed the equivalence of in vitro and in vivo data (Table 1) and suggested that the Bio-Plex assay would be applicable for use in tissue samples.

Table 1. Comparison of HCC827 cell lysate (in vitro) and xenograft (in vivo) Bio-Plex assay data. Values represent PI, percentage of total protein MFI represented by phosphorylated protein MFI. The similar fold decrease between in vitro and in vivo phosphorylation indices indicate Bio-Plex assays would be applicable for tissue samples.

	EGFR	ERK1/2	S6
Cell lysate (in vitro)			
+ EGF	387.3	67.3	76.5
+ EGF + gefitinib	25.1	22.1	28.3
Fold decrease	15.4	3.0	2.7
Xenograft (in vivo)			
Control	63.2	6.3	179.9
+ gefitinib	4.2	2.5	119.4
Fold decrease	15.0	2.5	1.5

Conclusions

The Bio-Plex assays and western blots detected total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein equivalently in HCC827 cell lysate. Both assays' results reflected a decrease in phosphorylation upon treatment with gefitinib in vitro.

When HCC827 xenograft tumors were evaluated by Bio-Plex assays and western blots, there was equivalent detection of total and phosphorylated EGFR, ERK1/2, and S6 ribosomal protein. There was a significant correlation between the Bio-Plex assay and western blotting results. In addition, there was statistically significant suppression of phosphorylation when mice were treated with the EGF inhibitor gefitinib compared to controls.

This study also demonstrated that the in vitro and in vivo HCC827 experiments gave equivalent results and that the Bio-Plex assay is not only useful for detecting phosphoproteins in cell lysates, but also in tumor tissue.

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