Using Bio-Plex® Phosphoprotein Assays to Study EGFR Signaling in Human Patient-Derived Xenografts Treated With Cetuximab

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Introduction
A hallmark of cancer is aberrant and unlimited cell proliferation. Activation of cell surface receptors such as the epidermal growth factor receptor (EGFR/HER1) plays an important role in promoting cell proliferation and tumor growth in certain cancer types. Constitutive activation of EGFR and downstream signaling pathways are therefore of high pathophysiological relevance (Hynes and Lane 2005). Cetuximab (trade name ERBITUX) is a monoclonal antibody that targets the extracellular domain of EGFR, thereby blocking ligand (EGF) binding and preventing both receptor activation and downstream phosphoprotein signaling cascades (Figure 1). Cetuximab has been used for treatment of a number of advanced cancers, including colorectal cancer, head and neck cancer, and non-small cell lung cancer. However, the drug is effective in only a fraction of the patients who receive treatment, and the mechanisms underlying primary resistance towards cetuximab are poorly understood (Arribas and Baselga 2005).

Methods

Lysate Preparation
Snap-frozen xenograft material was provided by the Oncotest in vivo facilities. Native tumor lysates were prepared using the Bio-Plex cell lysis kit and protocol (Bio-Rad).

Oncotest Lysate Pool
The Oncotest lysate pool was generated from lysates of 106 different patient-derived tumor xenografts and represents 21 different tumor entities (Table 1). This pool is used to measure expression of various proteins in tumor tissue. Normalization to the pool was used to minimize plate-to-plate variations and to estimate whether a value represented a high or low protein level by comparing it to the average protein level over the many tumor tissues represented in the pool.

Table 1. Cancers represented in the Oncotest lysate pool by organs/histotypes.

<table>
<thead>
<tr>
<th>Cancer Type</th>
<th>Number</th>
<th>Cancer Type</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bladder</td>
<td>1</td>
<td>Lymph</td>
<td>1</td>
</tr>
<tr>
<td>Blood, leukemia</td>
<td>2</td>
<td>Melanoma</td>
<td>9</td>
</tr>
<tr>
<td>Breast</td>
<td>11</td>
<td>Ovary</td>
<td>8</td>
</tr>
<tr>
<td>Cervix</td>
<td>2</td>
<td>Pancreas</td>
<td>4</td>
</tr>
<tr>
<td>Colon</td>
<td>13</td>
<td>Pleural mesothelioma</td>
<td>2</td>
</tr>
<tr>
<td>Head and neck</td>
<td>2</td>
<td>Prostate</td>
<td>4</td>
</tr>
<tr>
<td>Kidney</td>
<td>6</td>
<td>Sarcoma</td>
<td>3</td>
</tr>
<tr>
<td>Liver</td>
<td>2</td>
<td>Stomach</td>
<td>5</td>
</tr>
<tr>
<td>Lung, small cell</td>
<td>5</td>
<td>Testis</td>
<td>2</td>
</tr>
<tr>
<td>Lung, non-small cell</td>
<td>24*</td>
<td></td>
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</tr>
</tbody>
</table>

*Adenocarcinoma (15), epidermoid cancer (6), and large cell carcinoma (3).

To investigate cetuximab sensitivity and resistance, we studied its effects on the EGFR signaling pathway in 34 human patient-derived tumor xenografts developed at Oncotest GmbH (Freiburg, Germany). The Bio-Plex suspension array system (from Bio-Rad Laboratories, Inc.) was used for the detection of EGFR as well as Akt (PKB), a key downstream signaling protein involved in cell survival. Bio-Plex phosphoprotein assays were used to demonstrate that the effectiveness of cetuximab is not determined solely by its ability to inhibit EGFR activation.

Fig. 1. Targeting EGFR. Cetuximab binds the EGF receptor, preventing its activation by EGF and affecting the downstream signaling cascade that controls cell proliferation.
**Xenograft Models**

Xenografts were derived from non-small cell lung cancer (LXFA, 8 grafts; LXFE, 3 grafts; LXFL, 3 grafts), colon (CXF, 11 grafts), mammary (MAXF, 2 grafts), gastric (GXF, 1 graft), head and neck (HNXF, 6 grafts) cancers, and melanoma (MEXF, 1 graft).

**Data Collection and Analysis**

To better understand the effect of cetuximab on tumor growth and EGFR signaling, we treated xenograft-bearing nude mice on days 0, 7, and 14 with 30 mg/kg of the drug and measured tumor volumes typically until days 21–30. Levels of total and phosphorylated EGFR and Akt were determined in untreated tumors (control), as well as in tumors at 24, 48, and 72 hr after a single dose of cetuximab (Figure 2).

![Fig. 2. Experimental design. Tumor-bearing mice were treated intravenously with 30 mg/kg cetuximab. Tumors were collected for total and phosphoprotein analysis at various times.](image)

The starting sample amount was 12.5 μg of protein per assay point. Akt (Ser\(^{473}\)) and EGFR (Tyr) phosphoprotein assays and the Akt total target assay were from Bio-Rad. The total EGFR assay was purchased from a third-party vendor and applied with the Bio-Plex buffers (Bio-Rad). All measurements were done in duplicate and only included in this report if the %CV was <20. The Oncotest lysate pool was run as a normalization control at the start and at the end of every plate. Average median fluorescence intensity (MFI) values from the pool were set as 1, and the sample MFIs were normalized against the pool (nMFI) for each plate.

**Results**

In vivo tumor responses to cetuximab are depicted as a waterfall plot (Figure 3). Tumors with optimal treated to control (T/C) size ratios of ≤35% were considered highly responsive to cetuximab and those with T/C values >35% as weakly sensitive/resistant. Total EGFR expression and activation status in untreated tumors are shown in Figure 3A.

![Fig. 3. EGFR and Akt activation status and in vivo response to cetuximab.](image)
The data show that in selected tumors, high levels of expression and activation of EGFR correlate with a high response to cetuximab treatment as would be expected (HNXF2, GXF1). This indicates that growth of these tumors is driven by high levels of EGFR pathway activation. However, some tumors had relatively high levels of phosphorylated EGFR (LXFA5), but did not respond to cetuximab treatment. Analysis of EGFR activation in treated tumors indicates that cetuximab is effective at inhibiting EGFR activation both in tumors that are responsive and in tumors that are not responsive (Figure 4). Therefore, growth of unresponsive tumors may be driven by signaling pathways independent of EGFR activation.

Expression and activation of Akt (PKB) was also measured. Figure 3B shows the levels of Akt expression and phosphorylation in control xenografts in relation to cetuximab’s in vivo efficacy. Total Akt protein was expressed at similar levels across the panel of tumors, but activation levels varied. Xenograft models with highly active Akt signaling were among those that did not respond well to cetuximab treatment (LXFE3, CXF8, MAXF2).

**Discussion and Conclusions**

This study on the effect of cetuximab on EGFR signaling shows that using the Oncotest lysate pool as a control makes it possible to rate the activation and expression status of biologically relevant proteins. Comparing to the average of 106 different tumors with 21 different histologies allowed identification of tumors with a broad range of EGFR/Akt activation levels.

The data show that high expression and activation of EGFR is more frequently observed in cetuximab-responsive tumors, whereas very high levels of Akt activation are found in cetuximab-unresponsive tumors. Reduction of phosphorylated EGFR by cetuximab treatment does not distinguish cetuximab-sensitive from cetuximab-resistant tumors.

Thus, Bio-Plex phosphoprotein assays, in combination with Oncotest’s patient-derived tumor xenograft lysates, can be used by researchers to probe deeper into the mechanisms of cancer therapeutics. These tools can help clinicians understand why even the best available treatments lack efficacy in some patients.

**References**


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