Effects of antibody-mediated EGF-receptor inhibition on ERK1/2 isoform phosphorylation in organoid cultures

Abstract # 3605

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Introduction

Targeted anti-cancer therapy using small molecules or therapeutic antibodies is important to improve the treatment options of individual cancer patients whose tumor show specific expression patterns of respective target proteins. In order to enhance the development of new targeted drugs novel and highly predictive *in vitro* drug testing models are needed which closely reflect the characteristics of each individual tumor.

Towards this end Indivumed has developed a preclinical drug testing platform based on freshly cultivated tumor tissue slices which enables a detailed investigation of functional effects of classical chemotherapeutic drugs, small molecules and therapeutic antibodies in a natural tumor microenvironment. In addition this multifunctional *in vitro* model permits the evaluation of target expression and analysis of signaling pathway activities.

The aim of the present study was to analyze and verify the functionality of an anti-EGFR antibody in colorectal cancer tissue slices using our recently developed drug testing platform. As readout of treatment effects changes in the expression and phosphorylation status of selected signaling proteins from two EGFR-related downstream pathways, the MAPK and Akt pathways, were evaluated by Meso Scale Discovery (MSD) assays and immunohistochemistry. To further analyze the complex regulation of phosphorylation pattern in more detail we integrated the new NanoPro 1000 technology in our pathway analysis, enabling the identification of distinct isoform phosphorylations. This approach should help to extend the knowledge about individual drug responses among patients to further advance personalized medicine.

Methods

Preparation, cultivation and treatment of tissue slices

Tumor tissue pieces from colorectal cancer (CRC) patients were collected immediately after resection according to Indivumed's standard operating protocols and subsequently used as starting material for the preparation of tissue slices. Tissues were cut into 400 µm slices using a Krumdieck™ tissue slicer (TSE Systems). Tissue slices were cultivated in a supplemented RPMI 1640 tissue culture medium in 24 well plates.

For antibody treatment CRC tissue slices were pre-cultured for one hour before the anti-EGFR antibody was added at three concentrations for 48 hours. Samples treated with a control IgG pool at a single concentration served as control.

Analysis of signaling pathways

Proteins from signaling pathways were analyzed by Meso Scale Discovery (MSD) assay, immunohistochemistry, and NanoPro1000 technology. For quantification of selected signaling proteins (total and phosphorylated Akt and ERK1/2 (MAPK)) via Meso Scale Discovery (MSD) assays , tissue slices were lysed with MSD lysis solution (MSD-kit, MSD) and protein concentration was determined by Bicinchoninic Acid Protein (BCA) assay (BCA kit, Sigma). Afterwards, tissue lysates in triplicates were subjected to the respective 96-well MSD plate and MSD assay was performed according to manufacturer's instruction.
Further analysis of ERK1/2 (MAPK) was conducted using the NanoPro1000 technology platform. This technology enables the identification of multiple isoforms according to their isoelectric point. Therefore, protein lysates were separated on a nested Premix G2 5-8 gradient against the pI standard ladder 3 and immobilized for 70 seconds.

Immunohistochemistry

Tumor tissue slices of each case and condition were formalin fixed, paraffin embedded and cut into five µm thick slices. For EGFR, Ki67 and pERK1/2 (pMAPK) staining slides were subjected to IHC using the Benchmark® Ultra (Roche Diagnostics Deutschland GmbH). Stained sections were examined under microscopy.

Workflow & Technologies

Drug testing platform

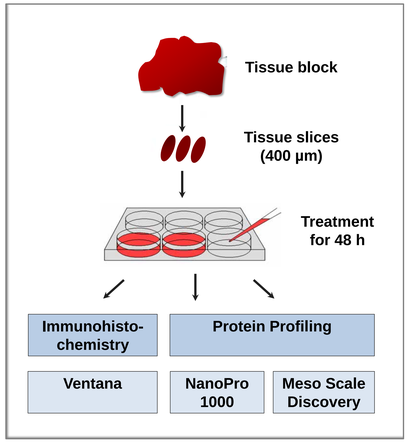

Figure 1: Drug testing platform
Flow chart showing the essential steps for tumor tissue slice preparation and cultivation. Freshly cultivated tissue slices can be treated with drugs for up to 72 hours. Comprehensive analysis of drug responses can be conducted using several readouts.

NanoPro1000 technology

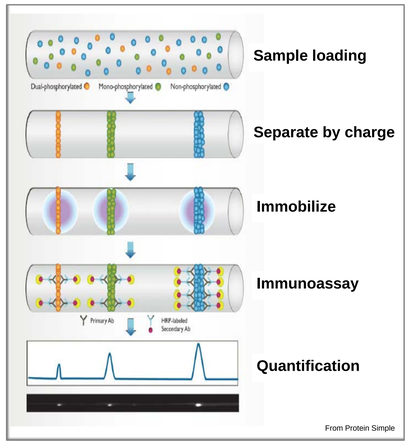

Figure 2: NanoPro1000 technology
The NanoPro 1000 Technology enables the identification of multiple isoform phosphorylations by separating low amounts of proteins according to their isoelectric point. Therefore, by integrating this technology into our drug testing platform we are able obtain more detailed information about activation and inactivation of signaling proteins.

Results

Effect of anti-EGFR antibody treatment on pathway activity of colorectal cancer tissue slices

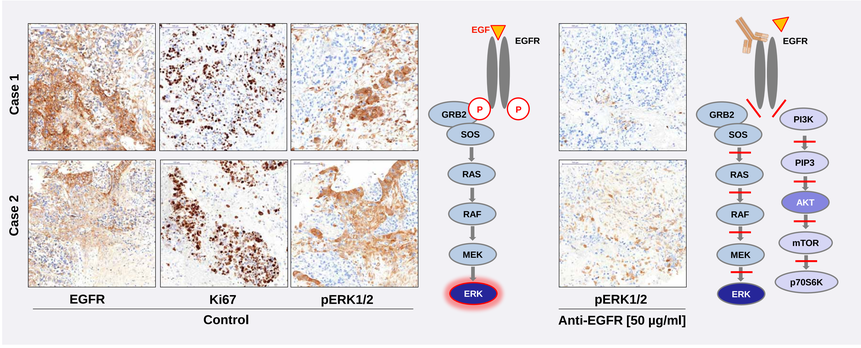

Figure 3: IHC staining results of control IgG and anti-EGFR antibody treated CRC tissue
Colorectal cancer (CRC) tissue slices were incubated with an anti-EGFR antibody [50 µg/ml] or a control IgG pool for 48 hours. IHC staining was performed against EGFR, Ki67 and pERK1/2 (pMAPK).
IHC staining revealed that protein expression patterns differed between individual patients, as shown here exemplarily for two selected cases. Case 1 demonstrated a strong EGFR expression whereas case 2 was only weak positive. Inhibition of EGFR by anti-EGFR antibody treatment resulted in a strong reduction of ERK1/2 phosphorylation, especially observed in case 1.

Inactivation of signaling proteins by anti-EGFR antibody treatment

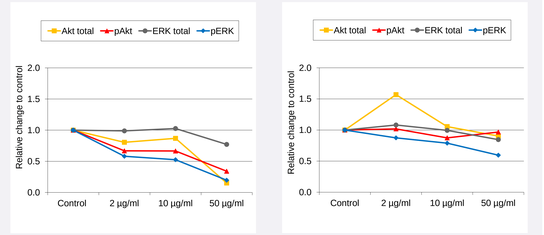

Figure 4: Expression levels of proteins from signaling pathways in anti-EGFR antibody treated CRC tissue
CRC tissue slices were treated with different concentrations of an anti-EGFR antibody or with a control IgG for 48 hours. Afterwards the expression levels of total and phosphorylated Akt and ERK1/2 were analysed by Meso Scale Discovery (MSD) assay. The relative change of expression levels (mean values) of proteins in antibody-treated tumor tissues to controls is shown here for the same two cases as in figure 3.
We observed individual drug responses upon different patients. Inhibitory drug effects were visible for case 1 showing a dose-dependent reduction of total and phosphorylated Akt and ERK1/2.

Individual ERK1/2 isoforms determined with NanoPro1000

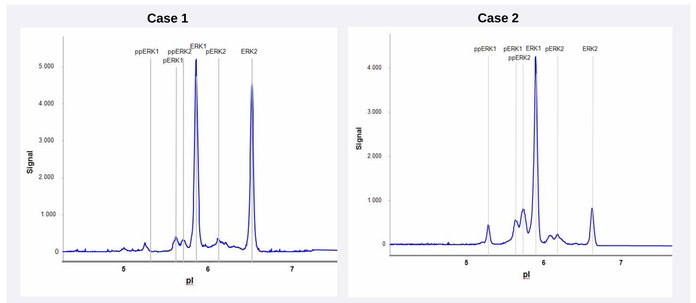

Figure 5: Individual isoform phosphorylation patterns of ERK1/2 in CRC tissues
Basal isoform phosphorylation patterns of ERK1/2 were evaluated in CRC tissue lysates using the NanoPro1000 technology. The ERK1/2 isoform pattern differed among patients, as exemplified here by case 1 and case 2, thus representing the heterogeneity in molecular signatures among patients.

Detection of ERK1/2 isoforms after anti-EGFR antibody treatment

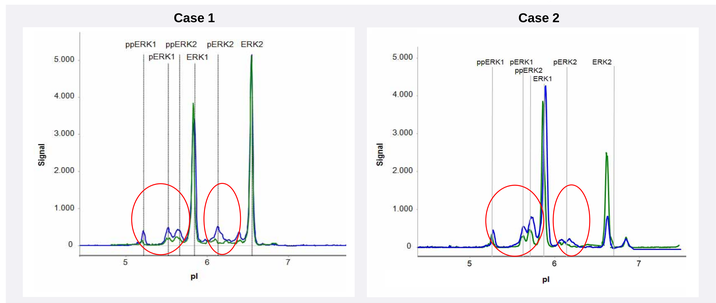

Figure 6: Regulation of ERK1/2 isoform phosphorylation in anti-EGFR antibody treated CRC tissue
Changes in the isoform phosphorylation patterns of ERK1/2 in anti-EGFR antibody [50 µg/ml] treated and control IgG treated CRC tissue slices of the same cases as described in figure 5 after 48 hours measured by NanoPro 1000.
Green: anti EGFR-antibody, blue: control

Summary

- **In this study we used our previously developed organotypic drug testing platform for a more detailed evaluation of antibody-mediated EGF-receptor inhibition in colorectal cancer tissue slices.**
- **We have demonstrated that functional drug effects of a monoclonal anti-EGFR antibody could be measured in organoid cultures.**
- **By the use of different technologies we have illustrated that the expression and phosphorylation patterns of signaling proteins differed between individual patients, representing a potential basis for the investigation of individual drug responses.**
- **We have shown that the NanoPro1000 technology is useful to identify individual protein isoform phosphorylation patterns in tumor cells and their regulations that may occur during drug treatment.**
- **Thus, the combination of Indivumed's organotypic drug testing platform and the NanoPro technology provides an innovative opportunity for more comprehensive preclinical studies performed in a natural tumor microenvironment to test new drugs that target signaling pathways, to evaluate and forecast drug responses and to identify predictive biomarker to further improve personalized medicine.**