Unger et al., Int J Cancer Immunol Immun 2015, 1: 101
http://dx.doi.org/10.15344/ijcii/2015/101

International Journal of
Cancer Immunology & Immunotherapy

Research Article Open Access

Drug Profiling in Precision Cut Cancer Tumor Slices: Analysis of Therapeutic Antibodies in Colorectal Cancer

Florian T. Unger*, Jana Krüger, Hartmut Juhl and Kerstin A. David

Drug testing and proteomics labs, Indivumed GmbH, Falkenried 88, Building D, Hamburg, Germany

Abstract

Background: The aim of this study was to define dose- and time-dependent therapeutic effects of one anti-EGFR antibody and one anti-IGF-1R antibody in colorectal cancer tissue samples.

Methods: Expression and phosphorylation levels of Akt/pAkt, pmTOR, pp70S6K, ERK1/2 (MAPK) and pERK1/2 (pMAPK) were used as readout of treatment effects and analyzed by Meso Scale Discovery (MSD) and NanoPro™ 1000 technique. Additionally, immunohistochemistry studies on expression and distribution of EGFR and IGF-1R, pAkt and pERK 1/2, and Ki67 were performed.

Results: The expression and phosphorylation pattern of Akt, pAkt, pmTOR, pp70S6K, ERK1/2 (MAPK) differed between individual cases illustrating the huge heterogeneity of tumors. Expression was reduced, elevated or not affected by drug-treatment. The expression and phosphorylation levels of key proteins correlated between the members of the same pathway and case, showing comparable tendencies in curve progressions and drug response. The proliferation parameter Ki67 remained stable in four of five cases even after treatment with anti-EGFR and anti-IGF-1R indicating that viability of the tissue was not affected by treatment. The IGF-1 receptor was present in three cases, two cases showed no immunoreactivity. P-Akt and p-MAPK expression levels decreased after anti-EGFR and anti-IGF-1R antibody treatment in three cases. In one case no treatment effects were detected. The NanoPro™ 1000 analysis confirmed the MSD results for ERK1/2 and gave additional information on the treatment induced regulation of isoform phosphorylation of ERK1/2.

Conclusion: In two of five colorectal cancer cases treatment of cultured tissue slices with anti-EGFR and anti-IGF-1R antibody resulted in a clear reduction of expression levels of key proteins from Akt and MAPK pathway. Although the number of cases being examined in this study was too small to be statistically significant, the results gave a hint that both antibodies were functional in cultured precision cut cancer tissue slices and the performed analysis might be promising for the prediction of optimized, individualized anti colorectal cancer therapy. Overall, these results demonstrated that the used drug testing platform allows determination of drug effects in a heterogeneous group of colorectal cancer patients.

Publication History:

Received: April 15, 2015
Accepted: July 15, 2015
Published: July 17, 2015

Keywords:

Precision cut tissue slices, Antibodies, EGFR, IGF-R, Colorectal cancer

Background

Colorectal carcinomas (CRC) are still the third leading cause of cancer-related death in the Western world and the most important malignancies of the gut. Current anti-cancer therapies are based on conventional chemotherapeutic drugs, small molecules or therapeutic antibodies. The latter are directed against key receptors of signal transduction cascades. Expression levels of these receptors, like Epidermal Growth Factor Receptor (EGFR) or Insulin-Like Growth Factor Receptors (IGF-R) are often elevated in CRC patients. Some approved therapies for advanced colorectal cancer rely on monoclonal antibodies targeting EGFR. Unfortunately, lack of response to two anti EGFR drugs, Cetuximab and Panitumumab, has been observed in some patients. In this context, existence of mutations of diverse downstream signal mediators, as for example KRAS, BRAF or PI3KCA, are known to play an important role in development of resistance to therapeutic antibodies [1]. Therefore, knowledge not only of the receptor status, the gene mutation statusbut also of the activity of intracellular pathways of a patient is crucial to provide optimal individualized therapy [2,3].

The development of mutations and resistance to anti-cancer drugs is a common problem, preventing successful therapy and diminishing treatment options. Thus, there is an urgent need to develop new and more effective therapeutic anti-cancer drugs comprising new classes of therapeutic agents like small molecules [4-6] and therapeutic monoclonal antibodies [7-9]. Most of these drugs are directed against specific cellular and molecular targets of signaling pathways, such as key receptors, or signaling proteins. However, a major problem in drug development for cancer treatment is the incapability to predict from preclinical assays, which depend usually on cancer cell lines, drug effects in a highly heterogeneous tumor with a complex cellular structure [10,11-18].

The here used precision cut cancer tissue culture system represents a more promising in vitro model which combines the advantages of maintaining the heterogeneous and complex 3D tissue architecture, cell viability, pathway activity, overall gene expression and natural individual tumor microenvironment [19-22]. The model is based on cultivated cancer tissue slices derived from diverse tumor entities which permits detailed drug testing in individual tumor microenvironments and genetic backgrounds. We have established a drug profiling platform that is based on tumor tissue instantly cultivated after its surgical removal. Using proprietary cultivation methods tumor tissue can be cultivated for up to 72 hours without significant changes in viability and its molecular profile. Therefore,

***Corresponding Author:** Dr. Florian T. Unger, Drug testing and proteomics labs, Indivumed GmbH, Falkenried 88, 20251 Building D, Hamburg, Germany, E-mail: unger@indivumed.com

Citation: Unger FT, Krüger J, Juhl H,David KA (2015) Drug Profiling in Precision Cut Cancer Tumor Slices: Analysis of Therapeutic Antibodies in Colorectal Cancer. Int J Cancer Immunol Immun 1: 101. doi: http://dx.doi.org/10.15344/ijcii/2015/101

this model allows to study drug effects in a preclinical model that represents as close as possible the molecular reality of cancers and, subsequently, the effects of anti-cancer agents in a heterogeneous group of patients.

The aim of this study was to examine drug effects, in precision cut cancer cultured tissue slices, using antibodies with known mode of action. Therefore, the dose- and time-dependent effects of two therapeutic antibodies, anti-EGFR and anti-IGF-R, were studied in tumor tissue of five colorectal cancer patients.

Activation of both receptors occurs by binding of their respective ligand and results in activation of the same downstream signaling pathways, including beside others the Mitogen-Activated Protein Kinase (MAPK)-pathway and the Akt-pathway [23]. The therapeutic effect of both antibodies was determined by evaluation of the expression and phosphorylation level of selected key mediator proteins from MAPK-pathway (ERK 1/2) and Akt-pathway (Akt, mTOR and p70S6K) using the Meso Scale Discovery (MSD®) platform. The resulting phosphorylation status was considered as indicator for pathway activity. To gain a deeper view in treatment induced regulations of signaling protein isoform phosphorylation, we used the NanoPro™1000 technology in samples of three patients. In addition, tissue slices were analyzed by immunohistochemistry (IHC) assays to evaluate the EGF and IGF-1R receptor status, the phosphorylation pattern of Akt and ERK1/2 (MAPK) and to prove tissue viability by determination of the expression level of proliferation marker Ki67.

Methods

Tissue and data collection

All patients gave written consent and institutional review board approval was obtained at the physicians association in Hamburg, Germany. Tumor tissues from 5 colorectal cancer patients with well (1) and poorly (4) differentiated CRC were collected by Indivumed's study nurses in the surgery room. In less than 15 min after completion of surgical resection, tissue samples were either snap frozen in liquid nitrogen, fixed in formalin to serve as reference material or transferred to ice cold tissue preservation medium [RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 % MEM-vitamins, 1 % penicillin/streptomycin, 0.2 % gentamicin, 5 µg/mL transferrin, 12.5 µg/mL fetuin, 20 µg/mL insulin, 10 % fetal calf serum (FCS)] and subsequently used as starting material for the preparation of fresh tissue slices and/or primary cells.

Precision cut cancer tissue slice culture

In order to obtain tissue slices cultures, fresh tissue samples were cut into 400 µm x 5 mm thick tissue slices using Krumdieck™ tissue slicer MD4000-01 (TSE Systems, Bad Homburg, Germany) according to manufacturer's instructions. Cancer tissue slices were cultured in 24-well plates with tissue culture medium [RPMI 1640 medium supplemented with 2 mM L-glutamine, 25 nM sodium selenite, 10 µM ethanolamine, 10 µM phosphorylethanolamine, 100 pM triiodothyronine, 0.5 mM sodium pyruvate, 1 % MEM-vitamins, 1 % penicillin/streptomycin, 0.1 % gentamicin, 0.5 µg/mL fungizone, 10 µg/mL transferrin, 2 mg/mL bovine serum albumin (BSA), 1 ng/mL epidermal growth factor (EGF), 12.5 µg/mL fetuin, 20 µg/mL insulin and 0.5 mM hydrocortisone, and 5 % FCS] at 37°C and 5% CO^2. Additionally, plates were shaken at 100 rpm.

Drug testing platform

CRC tissue slices were incubated for 1 h before treatment with different antibodies. Anti-EGFR antibody and anti-IGF-1R antibody (Roche) at 3 different concentrations (2 µg/mL, 10 µg/mL, 50 µg/mL) and one control IgG (IgG, 150 µg/mL, Rabbit IgG, clone DA1E, Cell Signaling) for 24 h and 48 h were used. For each case and condition 5 tissue slices were used. Proliferation, expression and phosphorylation status of signal proteins from 2 EGFR- and IGF-1R-related pathways measured by MSD® and IHC were used as readout to monitor the effects of the antibodies.

Antibody diffusion ssay and immunofluorescence

Tissue slices were cultured in RPMI 1640 tissue culture medium for 1 h before incubation with the primary antibody, using either a mouse-anti-EGFR CY3 antibody (50 µg/ml and 250 µg/mL, 24, Roche) or a mouse-anti-IGF-R-1 CY3 antibody (150 µg/ml and 750 µg/ml, 24, Roche). Slices were then washed in PBS, frozen in liquid nitrogen, cut into 5 µm thick slices using the Cryostat Microm HM500 0 (Microm, Gudenrath, Germany) and fixed in 2% paraformaldehyde. Tissue slices were embedded in Fluoromount G (Southern Biotech, Birmingham, US) and components were visualized by fluorescence microscopy (fluorescence microscope, Zeiss, Gottingen). Untreated tissue slices served as control.

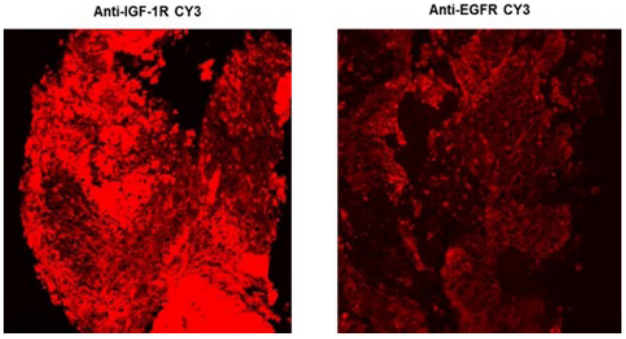

Figure 1: Immunofluorescence staining of CRC precision-cut tissue slices: CRC tissue slices from patient B1642 were incubated in full growth medium with mouse-anti-EGFR CY3 antibody (50 µg/mL and 250 µg/mL, 24 h) or a mouse-anti-IGF-1R CY3 antibody (150 µg/ml and 750 µg/ml, 24 h).

H & E Staining

Tumor slices from time point zero and from each case and condition were formalin fixed, embedded in paraffin and cut (5µm thickness). Resulting FFPE slices were mounted onto glass slides, dried for 30 min at 58°C, deparaffinized, heamatoxylin and eosin stained (H&E), dehydrated and sealed with cover glasses. Microscopy on stained sections was performed and tumor content was determined.

Preparation of tissue lysate

Tissue lysates were prepared using phospholysis buffer [containing 20mM Tris-HCl, 150mM NaCl, 5mM EDTA, 1% Triton X 100, Complete protease inhibitor cocktail (Roche, Mannheim, 1:7) and phosphatase inhibitor cocktail (Sigma, Steinheim, 1:100)] or MSD®lysis buffer (Gaithersburg, MD, USA). After 24 h or 48 h of treatment, respectively, 3 tissue slices from each condition were frozen in liquid nitrogen, subjected to lysis buffer and homogenized in a swing mill (MM300, Retsch, Haan, Germany) for 2 min at 30 Hz followed by incubation at 4°C for 30 min. Afterwards, lysates were centrifuged at 8'000 x g and 4°C for 10 min (Universal 32R centrifuge, Hettich, Tuttingen, Germany). For preparation of cell lysates cells were harvested and washed twice with D-PBS. Afterwards cells were re-suspended in lysis buffer and incubated for 30 min at 4°C. Finally, lysates were centrifuged at 8'000 x g for 10 min and 4°C (Universal 32R

centrifuge, Hettich, Tuttingen, Germany). Protein concentration was determined by Bicinchoninic Acid Protein assay (Sigma, Steinheim, Germany).

Production of control lysates and MSD® assays

For protein analysis antibody based MSD® assays were performed. MSD® plates with respective immobilized capture antibodies against total and phosphorylated proteins were used. In order to account for inter-plate differences, lysates of stimulated human cells were produced and employed as positive and negative controls according to manufacturer's instructions. Briefly, cell lines were grown according to standard conditions for recommended cell lines (Jurkat and HEK293), treated with respective agents and cell suspension was obtained.

Quantification of proteins was conducted using 96-well format technology platform from Meso Scale Discovery (MSD®, Gaithersburg, MD, USA).

MSD® 96-well MULTI-SPOT® assays were performed targeting key proteins by using antibodies against following proteins (total and/or phosphorylated): MAP kinase pathway [ERK1/2 (MAPK) / pERK1/2 (pMAPK) (Thr202/Tyr204, Thr185/Tyr187) duplex], Aktsignaling pathway [Akt / pAkt (Ser473) duplex, pmTOR (Ser 2448) singleplex, pp70S6K (Thr421, Ser424) singleplex].

The MSD® assay was performed using 10 µg of tissue lysate. Plates were read using MSD® SECTOR™ Imager (Meso Scale Discovery, Gaithersburg, MD, USA) for analysis. Evaluation of resulting data was performed according to manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry (IHC) protocols for EGFR, IGF-1R, Ki67, phosphorylated ERK1/2 (MAPK) and phosphorylated Akt were provided by Roche. Two treated tumor tissue slices of each case and condition were formalin-fixed, paraffin embedded and cut to 5 µm slices using the HM340 E rotation microtome (Microm, Walldorf, Germany). Slices were mounted onto glass slides and dried overnight at 56°C. For EGFR, IGF-1R, Ki67 and pERK1/2 (MAPK) staining slides were subjected to IHC using Benchmark® Ultra (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) whereas pAkt staining was performed by manual procedure. Following primary antibodies were used for IHC and diluted as described: Phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204) [diluted to 1:750, Cell Signaling (#4370-S)], Phospho-Akt (Ser473) (D9E) [diluted to 1:30, Cell Signaling (#4060-L)], EGFR [ready to use (RTU), Roche (#05278341001)], IGF-1R (G11) [RTU, Roche (#05278449001)], Ki-67 (30-9) [RTU, Roche (#5278384001)].

After staining, sections were treated with ascending ethanol series and xylene and covered with Pertex (Medite GmbH, Burgdorf, Germany). All samples were evaluated by light microscopy and scored based on the H-score. This score considers the staining intensity combined with the percentage of positive stained cells into the tumor tissue. The formula to obtain the score is as follows: 3-times the percentage of strongly stained tumor cells + 2-times the percentage of moderately stained tumor cells + 1-time the percentage weakly stained tumor cells. The score ranges from 0 to 300 with following results: 0-50 negative, 51-100 weak positive, 101-200 moderate positive, 201-300 strong positive results.

Diffusion of antibodies in colorectal tumor tissue

The homogeneity of antibody penetration into cultured tissue slices was analyzed. Therefore either fluorescently labelled anti-EGFR- or anti-IGF-1R antibody at two selected concentrations (representing either expected serum peak concentration in patients or maximum levels used in GLP-Tox studies) were subjected to tumor slices from two colorectal cancer patients and incubated for either 24 h or 48 h. Microscopically analysis showed a complete infiltration of both antibodies with variations of fluorescence intensity dependent on antibody concentration (Figure 2). For anti-EGFR antibody saturated signals could be observed in tumor cells and in the non-malignant compartment, whereas anti-IGF-1R antibody demonstrated massively saturated (over stained) signals in tumor cells and in the non-malignant compartment. Both antibodies penetrated into cultured tissue slices. Thus, functionality of therapeutic antibodies should be proven in tissue slice cultures.

Influence of EGFR and IGF-1R inhibitors in colorectal cancer tissue slices

Immunohistochemical staining of 5 patient samples showed different EGFR staining intensity, ranging from weak to strong staining (strongest H-score: 165, Figure 2, B, left upper panel). Immunohistochemical staining revealed in 2 cases no (H-scores: 0, data not shown) and in 3 cases weak to moderate (strongest H-score: 140, Figure 2, A, left upper panel) IGF-1R staining intensity.

Samples from patient B1642 treated with control IgG as well as with anti-EGFR and anti-IGF-1R showed strong (after 24 h) to moderate (after 48 h) positive staining, suggesting the presence of the EGFR receptor in this tissue (Figure 2A and B, upper panel). Control samples as well as anti-EGFR and IGFR treated samples showed moderately positive staining. Only after 48 h of treatment the immunoreactivity for IGF-1R was lower. Low expression of p-Akt was detectable in control samples treated for 24 h with IgG (Figure 2B, middle panel), after 48 h controls as well as treated samples were almost negative (score 10).Control samples showed a weak positive staining similar after 24 h and 48 h. Treatment with anti-EGFR and anti–IGF-1R lead to a decrease of pMAPK after 24 h.After 48 h of treatment with anti-EGFR and anti–IGF-1R a significant decrease was observed with results ranging from 50 to 0 (according to H- score negative reaction).

For Anti-EGFR, controls as well as treated samples showed a weak to moderate expression of the EGF receptor in patient sample R428 (Table 1). Anti-IGF-1R were evaluated as negative due to fact that IgG control samples as well as treated samples showed only single positive cells (Table 1).

Control samples cultivated for 24 h were weakly stained for p-Aktand negative after 48 h of cultivation. Treatment with anti-EGFR resulted in a slight decrease in p-Akt expression after 24 and 48h. Data in anti-IGF-1R treated specimen did not show a clear trend (Figure 2).Control samples revealed only a weak staining for p-MAPK after 24h and negative staining after 48 h. Treatment with anti–EGFR and –IGF-1R showed a slight decrease of p-MAPK in all samples after 24 h (Figure 2).

MSD®Assay for determination of inhibitory effects on pathway activities in antibody-treated colorectal cancer tissue

Phosphorylation level of ERK1/2 (MAPK), Akt, mTOR, and p70S6K was determined by Meso Scale Discovery (MSD®).The resulting phosphorylation status was considered as indicator for pathway activity, in which only proteins with a ≤ 0.5-fold expression level were regarded as significantly reduced. Reduction of phosphorylation

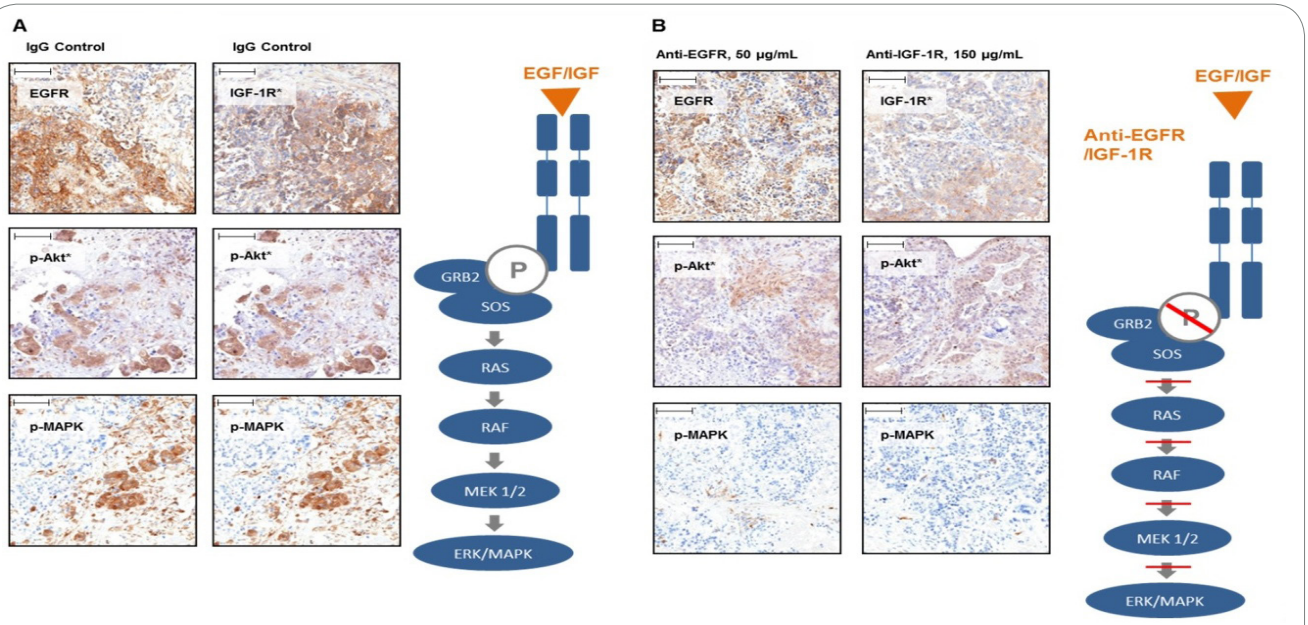

Figure 2: Immunohistochemical staining of CRC precision-cut tissue slices (patient B1642): CRC tissue slices were incubated in full growth medium with either (A) IgG control or (B) anti-EGFR, or anti-IGF-1R for 48 h. Immunohistochemical staining for EGFR (H-scores: 165 and 99), p-Akt (H-scores: 90 and 52), and p-MAPK (H-scores: 70 and 0) showed reduced expression when tissue slices were treated with anti-EGFR inhibitor. IGF-1R (H-scores: 140 and 105), p-Akt (H-scores: 90 and 57), and p-MAPK (H-scores: 70 and 0) showed reduced expression when tissue slices were treated with anti-EGFR inhibitor. Blue: haematoxylin, scale bars: 100µm, a representative staining result is shown.

Patient ID	Antigen	Staining Result
R428	IGF-1R	negative
	EGFR	weak positive
	Ki67	Stable after treatment
	p-Akt	Decrease after treatment (↓)
	p-MAPK	Decrease after treatment (↓)
R431	IGF-1R	negative
	EGFR	Moderate positive
	Ki67	Stable after treatment
	p-Akt	Stable at 24 h treatment, result not usable after 48 hours (bad preservation)
	p-MAPK	Stable but negative
B1640	IGF-1R	weak positive
	EGFR	Moderate positive
	Ki67	Stable after treatment
	p-Akt	Strong decrease after treatment
	p-MAPK	Strong decrease after treatment
B1642	IGF-1R	Moderate positive
	EGFR	Strong positive
	Ki67	Stable after treatment
	p-Akt	Strong decrease after treatment (↓) result not usable after 48 hours (bad preservation)
	p-MAPK	Strong decrease after treatment(↓)
B1643	IGF-1R	Moderate positive (↓)
	EGFR	Moderate positive (↓)
	Ki67	decrease after treatment (↓)
	p-Akt	decrease after treatment (↓)
	p-MAPK	decrease after treatment (↓)

Table 1: Histopathological evaluation of IHC stainings of the five colorectal cancer patients.

level of target protein in antibody-treated tumor tissue in relation to control IgG indicated potency of the antibody in the respective patient sample.

The Akt pathway can be activated indirectly by phosphorylation of the serine/threonine protein kinase Akt which thereupon mediates signal transduction. In its activated, phosphorylated form Akt phosphorylates a wide range of intracellular target proteins leading for example to activation of mTOR which activates p70S6-kinase. Thus different cellular processes like:(1) inhibition of apoptosis, (2) stimulation of cell proliferation and (3) stimulation of cell growth are regulated by Akt pathway. The key proteins examined here are Akt and pAkt, pmTOR and pp70S6K.MSD® analysis revealed inhomogeneous expression pattern for most of the target proteins differing between the individual patients (and Figure 3). Dependent upon the patient sample, protein expression was reduced, elevated or not affected by drug-treatment. However, for each patient correlations between the expression and phosphorylation levels of proteins of the same signaling cascade (Akt, pAkt, pmTOR and pp70S6K) could be observed, showing comparable tendencies in curve progressions and drug-response. This was the case in anti-EGFR and anti-IGF-1R antibody-treated tissue samples. However, patient 1642 showed reduced activity levels for all proteins (Figure 4A). Expression of Akt, pAkt, pmTOR and pp70S6 was diminished in a dose-dependent manner in anti-EGFR antibody-treated samples, leading to a more than 50 % reduction of pAkt and pp70S6 after 24 h and 48 h and of Akt and pmTOR after 48 h treatment with the highest drug concentration. In contrast, treatment with anti-IGF-1R antibody and resulted in a dose-dependent reduction of all proteins after 24 h, showing a more than 50 % decrease of pAkt at the middle and highest drug concentration (Figure 3A). Furthermore, a time-dependent but dose-independent reduction of Akt, pAkt, pmTOR and pp70S6 was visible after 48 h. At this time point expression levels of all proteins were significantly diminished below 50 %. Similar observations could be made for target proteins of patient B1643 (Figure 3B and Figure 4B). In this case expression levels of Akt, pAkt, pmTOR and ppS70K were diminished in a dose-dependent manner after treatment with anti-EGFR and anti-IGF-1R antibody for 24 h. Additionally, a time-dependent inhibitory effect was visible for some proteins at the lowest (Akt and pmTOR) and highest (Akt, pAkt and pp70S6K) anti-EGFR antibody concentration. A time-dependent reduction of expression levels could also be demonstrated for Akt, pAkt and pmTOR after 48 h treatment with anti-IGF-1R antibody (all concentrations), in which Akt and pAkt levels decreased significantly below 50 %. Phosphorylated ERK decreased dose-dependently after 48 hours in patient samples from R428 (Figure 3C and Figure 4C).

The mitogen-activated protein (MAP) kinase pathway is a signal transduction cascade which can be activated indirectly by phosphorylated EGFR or IGF-1R leading to phosphorylation and activation of downstream signal mediators like KRAS, BRAF, MEK and ERK1/2 (MAPK). Activated ERK1/2 phosphorylates cytoplasmic substrates and can also translocate to the nucleus where it causes phosphorylation of transcription factors regulating, amongst others, cell survival and growth. The key proteins of the MAP kinase pathway examined in this study are total and phosphorylated ERK1/2 (MAPK). Figure 3 and Figure 4 illustrate the relative change of expression levels of total and phosphorylated ERK in antibody treated tumor tissue in relation to control IgG. Reduced expression levels in anti-EGFR antibody treated samples could only be observed for pERK. Phosphorylated ERK decreased dose-dependently after 48 hours in patient R428 and after 24 h and 48 h in cases B1643 and B1642. However, a significant reduction (expression level ≤0.5-fold) could only be demonstrated for case B1642 at the middle (24 h) and highest (24 hand 48 h) drug concentration. Treatment with anti-IGF-1R antibody resulted in declining ERK and pERK levels in some cases. Expression of ERK was diminished in case B1643 after 24 h and 48 h and pERK levels decreased ~50 % within 48 h (Figure 3B). A reduction of pERK could also be observed in cases R428 after 48 h treatment with anti-IGF-1R antibody (Figure 3C). However, case B1642 represented the most interesting case, demonstrating a dose-dependent decrease of ERK after 24 h and 48 h and of pERK after 24 h. Furthermore, a time-dependent but dose-independent significant reduction of pERK was visible after 48 h, reaching expression levels below 50%.

Isoform phosphorylation determined by NanoPro™ 1000

To obtain a complex pattern of phospho-isoforms, an analysis onERK1/2 has been performed using the NanoPro™ 1000 technology. This new method allows the detailed identification of distinctisoform phosphorylations by separating the proteins according o their isoelectric point (pI). The regulation of isoform phosphorylation patterns of ERK1/2differed after treatment among patients, confirming the heterogeneity of individual responses among patients. As before, a dose-dependent regulation of total and phosphorylated ERK1/2was observable in the tissue slice cultures after anti-EGFR antibody treatment, exemplary shown by reduced isoform phosphorylation levels (Figure 5). Data of three patients (B1642, B1643 and R428) are shown in table 2.

Discussion

In this study, dose- and time-dependent therapeutic effects of two different antibodies were analyzed in colorectal cancer tissue samples freshly obtained from surgical specimen of five patients. Antibodies were either directed against EGFR or IGF-1R, two receptors that are of major clinical interest concerning targeted colorectal cancer therapy based on therapeutic antibodies. Cell proliferation as well as expression and activity levels of selected key mediator proteins of two EGFR- and IGF-1R-related signaling pathways were used as readout of treatment effects of the two different antibodies. Analysis were performed by Meso Scale Discovery (MSD®) technology platform and immunohistochemistry assay (IHC). In addition, H&E staining of FFPE tumor slices from one tissue section per case was performed to assure presence of tumor cells in tissue samples.

The ultimate goal was to determine and verify drug effects of therapeutic antibodies using an in vitro model based on precision cut cancer tissue slices, that represent the heterogeneity of tumors and their complex cellular structure as close as possible to the clinical situation. Thus, two aspects should be proven in this study, first of all, applicability of the drug profiling platform and secondary, functionality of therapeutic antibodies.

Colorectal cancer tissue slices of five patients were cultivated and treated with anti-EGFR or anti-IGF-1R antibody using three different concentrations for 24 and 48 hours.

Evaluation of MSD derived activity levels of selected target proteins in antibody treated tumor samples revealed that the expression pattern of Akt, pAkt, pmTOR, pp70S6K, ERK (MAPK) and pERK differed between the individual cases. This finding illustrated the high heterogeneity of tumors and differences between individual patients. However, the expression and phosphorylation levels of key proteins

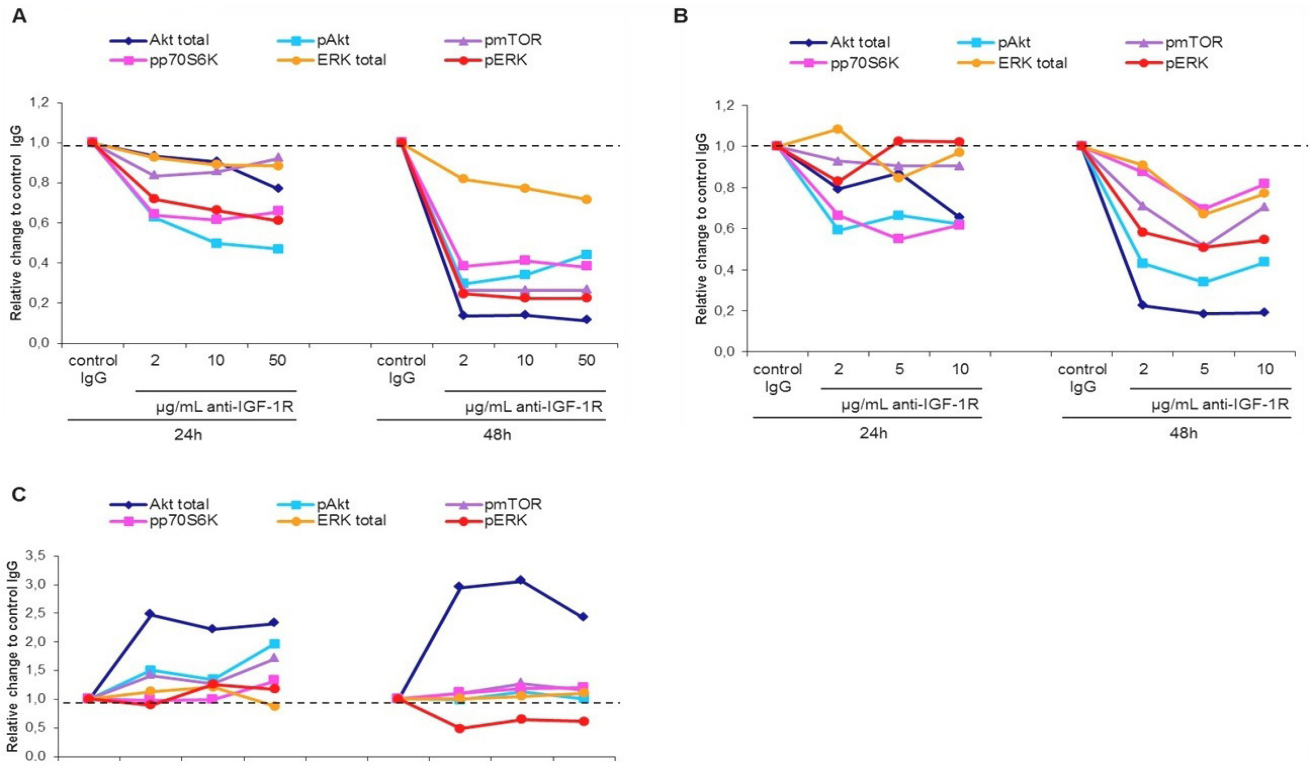

Figure 3: Expression and phosphorylation levels of signal proteins in anti-IGF-1R treated CRC tissue slices measured by MSD®: Expression levels (mean values) of total and phosphorylated Akt, phosphorylated mTOR and p70S6K as well as total and phosphorylated ERK (MAPK) in colorectal cancer tissue (A) patient 1642 (B) patient 1643 (C) patient R428. Tissue slices were treated with different concentrations of anti-IGF-1R antibody or control IgG for 24 or 48 hours. The relative change of expression levels (mean values) of proteins in antibody-treated tumor tissue to control IgG is shown.

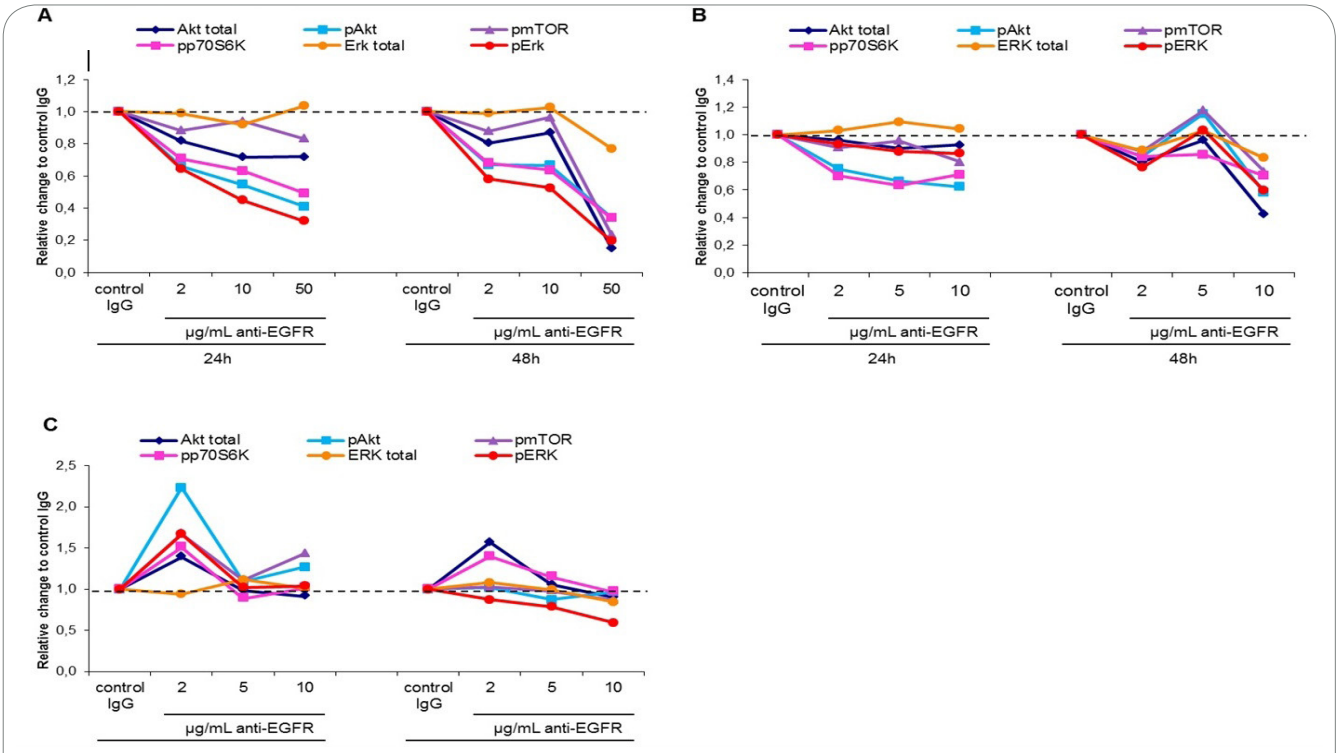

Figure 4: Expression levels of signal proteins in anti-EGFR treated CRC tissue slices measured by MSD®: Expression levels (mean values) of total and phosphorylated Akt, phosphorylated mTOR and p70S6K as well as total and phosphorylated ERK (MAPK) in colorectal cancer tissue (A) patient 1642 (B) patient 1643 (C) patient R428. Tissue slices were treated with different concentrations of anti-EGFR antibody or control IgG for 24 or 48 hours. The relative change of expression levels (mean values) of proteins in antibody-treated tumor tissue to control IgG is shown.

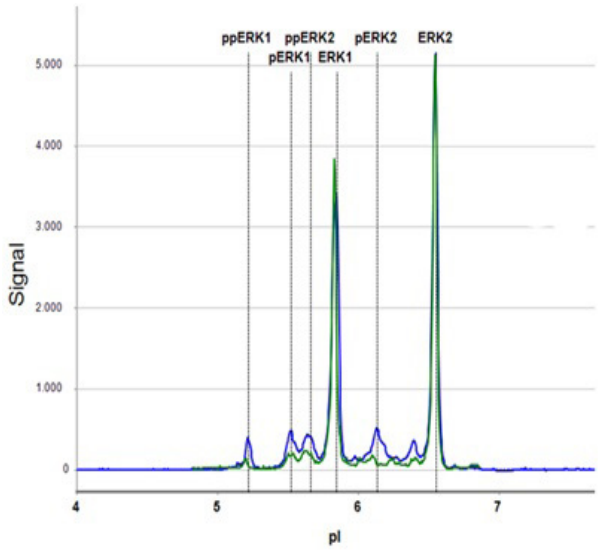

Figure 5: Exemplary spectra for the analysis of isoform phosphorylation pattern of ERK1/2 in CRC tissue lysates determined by NanoPro™ 1000: Changes of the isoform phosphorylation pattern of ERK1/2 in anti-EGFR antibody treated (50 µg/ml, green) and control (treated with 150 µg/ml control IgG pool, blue) CRC tissue slices of the same patient after 24h treatment. pI: isoelectric point.

Patient	Treatment	ERK1	pERK1	ppERK11	ERK2	pERK2	ppERK2
B1642	Control	79.94	14.86	5.20	66.04	16.10	17.87
	2 µg/ml anti- EGFR	86.09	9.01	4.92	62.68	16.51	20.82
	5 µg/ml anti- EGFR	89.38	7.08	3.54	57.71	22.48	19.82
	10 µg/ml anti- EGFR	84.97	10.75	4.29	20.09	73.50	6.42
B1643	Control	87.61	8.01	4.40	66.71	2.39	30.90
	2 µg/ml anti- EGFR	90.59	7.48	1.95	42.14	6.90	50.96
	5 µg/ml anti- EGFR	89.93	7.77	2.30	65.94	5.46	28.60
	10 µg/ml anti- EGFR	88.13	8.10	3.78	37.11	12.55	50.34
R428	Control	81.37	13.83	4.80	47.07	13.10	39.84
	2 µg/ml anti- EGFR	85.38	11.10	3.53	81.81	9.53	8.66
	5 µg/ml anti- EGFR	84.58	11.42	4.01	82.31	8.56	9.05
	10 µg/ml anti- EGFR	87.40	9.79	2.82	83.63	6.74	9.63

Table 2: Results from the NanoPro™1000 analysis of treatment induced changes in isoform phosphorylations for three patients. Percentage of AUC for ERK1 (ERK1=100%) and percentage of AUC for ERK2 (ERK2=100%) are displayed.

correlated between the members of the same pathway and case, showing comparable tendencies in curve progressions and drug response. For two cases, B1642 and B1643, strong inhibitory drug effects could be observed after treatment with anti-EGFR and anti-IGF-1R antibody and both antibodies diminished the expression and phosphorylated levels of key proteins from Akt and MAPK pathway. Especially case B1642 showed a dose-dependent reduction of Akt, pAkt, pmTOR, pp70S6K, ERK and pERK in anti-EGFR antibody treated tumor tissue samples. The same proteins decreased in a dose- and time-dependent manner after treatment with anti-IGF-1R antibody. In addition, it could be shown that the expression levels of all these proteins, except ERK, were significantly reduced below 50% by 48 hours at the latest in anti-EGFR and anti-IGF-1R treated samples of case B1642.

Immunohistochemistry (IHC) studies on pAkt and pERK1/2 (pMAPK) were performed to validate MSD data. Expression pattern demonstrated that both signaling proteins decreased after anti-EGFR and anti-IGF-1R antibody treatment in cases B1640, B1642 and B1643. This finding confirmed MSD data obtained for cases B1642 and B1643, underlining the functionality and inhibitory effects of both antibodies.

Since a therapeutic antibody is directed against its specific target receptor, knowledge about the receptor status of the tumor tissue is essential for successful anti-cancer treatment. Therefore, IHC analyzes on EGF- and IGF-receptor status were conducted. Resulting staining scores revealed that EGFR was present in all cases, showing the strongest expression in case B1642. IGF-1R was only expressed in cases B1642 and B1643 and weakly in case B1640. The evaluated receptor status of cases B1642 and B1643 were in accordance with the observed drug response represented by decreasing key protein expression levels measured by IHC and MSD®.

IHC staining of the proliferation marker Ki67 demonstrated that proliferation and tissue viability remained stable in 4/5 cases. However, in one of the receptor expressing cases treatment reduced Ki67 expression indicating also an anti-proliferative effect on the tumor. Overall, these results demonstrate that the used drug testing platform allows determination of drug effects in a heterogeneous group of colorectal cancer patients. Although the number of cases being examined in this study was too small to be statistically significant; the results gave a hint that both antibodies might be promising drugs for targeted and individualized colorectal cancer therapy.

Conclusion

In summary, the data demonstrated that functional drug effects of a monoclonal anti-EGFR and ananti-IGF-1R antibody could be measured in precision-cut tissue slice cultures. Therefore, the preclinical model based on cultured cancer tissue slices is suitable for examining the effects not only of classical chemotherapeutics and small molecules such as kinase inhibitors as we have shown recently [22], but also of larger molecules, such as therapeutic antibodies. The results suggest that the model based on tissue culturesis suitable for a more comprehensive analysis of drug responsesand in particular cellular responses to targeted drugs in a natural tumor microenvironment. Furthermore, the MSD® and NanoPro™1000 technology can potentially be useful for identifying individual

phosphorylation patterns of tumor cells, which may facilitate the discovery of predictive biomarkers for targeted therapies to further improve personalized anti-cancer therapy. For the newly introduced approach of immunotherapy, using immune-modulatory compounds, which target specific epitopes on cancer cells and recruit immune cells for anti-cancer effects, it is of great importance to get an impression on the epitope expression as well as immune cell infiltration of individual tumors. Due to the ability to perform IHC on the cancer tissue slices, we are able perform double staining for the determination of target epitopes and various immune cell markers.

Initial experiments also showed that immune cells are present within the tissue slices. However, it has not yet been comprehensively shown that this platform is able to evaluate the functions, actions of immunocytes and further experiments are needed.

Competing Interests

The authors have declared that no competing interests exist.

Author Contributions

JK and FTU carried out the immunoassays.
KAD and FTU conceived of the study and participated in coordination, performed the statistical analysis and drafted the manuscript.
HJ participated in the design of the study.
All authors read and approved the final manuscript.

Acknowledgements

We acknowledge Roche Diagnostics GmbH for providing therapeutic antibodies and Dr. Poßögel for pathological evaluation of biospecimen.

References

1. Tol J, Dijkstra JR, Klomp M, Teerenstra S, Dommerholt M, et al. (2010) Markers for EGFR pathway activation as predictor of outcome in metastatic colorectal cancer patients treated with or without cetuximab.Eur J Cancer 46: 1997-2009.
2. Dattatreya S (2013) Metastatic colorectal cancer-prolonging overall survival with targeted therapies.South Asian J Cancer 2: 179-185.
3. Li C (2014) A targeted approach to cancer imaging and therapy.Nat Mater 13: 110-115.
4. Workman P, Clarke P1 (2012) PI3 Kinase in Cancer: From Biology to Clinic. Am Soc Clin Oncol Educ Book.
5. Sahu A, Prabhash K, Noronha V, Joshi A, Desai S (2013) Crizotinib: A comprehensive review.South Asian J Cancer 2: 91-97.
6. Rosen LS, Gordon MS, Robert F, Matei DE (2014) Endoglin for targeted cancer treatment.Curr Oncol Rep 16: 365.
7. Burris HA (2012) Trastuzumab Emtansine (T-DM1): Hitching a Ride on a Therapeutic Antibody.Am Soc Clin Oncol Educ Book .
8. Shin TH, Sung ES, Kim YJ, Kim KS, Kim SH, et al. (2014) Enhancement of the tumor penetration of monoclonal antibody by fusion of a neuropilin-targeting peptide improves the antitumor efficacy.Mol Cancer Ther 13: 651-661.
9. Overington JP, Al-Lazikani B, Hopkins AL (2006) How many drug targets are there?Nat Rev Drug Discov 5: 993-996.
10. Roskelley CD, Bissell MJ (2002) The dominance of the microenvironment in breast and ovarian cancer.Semin Cancer Biol 12: 97-104.
11. Huang M, Shen A, Ding J, Geng M (2014) Molecularly targeted cancer therapy: some lessons from the past decade.Trends Pharmacol Sci 35: 41-50.
12. Hensing T, Chawla A, Batra R, Salgia R (2014) A personalized treatment for lung cancer: molecular pathways, targeted therapies, and genomic characterization.Adv Exp Med Biol 799: 85-117.
13. Trédan O, Galmarini CM, Patel K, Tannock IF (2007) Drug resistance and the solid tumor microenvironment.J Natl Cancer Inst 99: 1441-1454.
14. Patocs A, Zhang L, Xu Y, Weber F, Caldes T, et al. (2007) Breast-cancer stromal cells with TP53 mutations and nodal metastases.N Engl J Med 357: 2543-2551.
15. Orimo A, Weinberg RA (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type.Cell Cycle 5: 1597-1601.
16. Kinzler KW, Vogelstein B (1998) Landscaping the cancer terrain.Science 280: 1036-1037.
17. Bissell MJ, Radisky D (2001) Putting tumours in context.Nat Rev Cancer 1: 46-54.
18. Morin PJ (2003) Drug resistance and the microenvironment: nature and nurture.Drug Resist Updat 6: 169-172.
19. Schmeichel KL, Bissell MJ (2003) Modeling tissue-specific signaling and organ function in three dimensions.J Cell Sci 116: 2377-2388.
20. van der Kuip H, Mürdter TE, Sonnenberg M, McClellan M, Gutzeit S, et al. (2006) Short term culture of breast cancer tissues to study the activity of the anticancer drug taxol in an intact tumor environment.BMC Cancer 6: 86.
21. Guyot C, Combe C, Clouzeau-Girard H, Moronvalle-Halley V, Desmouliere A (2007) Specific activation of the different fibrogenic cells in rat cultured liver slices mimicking in vivo situations.Virchows Arch 450: 503-512.
22. Vaira V, Fedele G, Pyne S, Fasoli E, Zadra G, et al. (2010) Preclinical model of organotypic culture for pharmacodynamic profiling of human tumors.Proc Natl Acad Sci U S A 107: 8352-8356.
23. Cohen SJ, Cohen RB, Meropol NJ (2005) Targeting signal transduction pathways in colorectal cancer--more than skin deep.J Clin Oncol 23: 5374-5385.