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Original Article

Simultaneous inhibition of IGF1R and EGFR enhances the efficacy of standard treatment for colorectal cancer by the impairment of DNA repair and the induction of cell death

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ABSTRACT

Overexpression and activation of receptor tyrosine kinases (RTKs), such as the insulin-like growth factor 1 receptor (IGF1R) and the epidermal growth factor receptor (EGFR), are frequent phenomena in colorectal cancer (CRC). Here, we evaluated the effect and the cellular mechanisms of the simultaneous inhibition of these two RTKs both *in vitro* and *in vivo* in addition to a 5-fluoruracil (5-FU)-based radiochemotherapy (RCT), which is a standard treatment scheme for CRC. Using the small molecule inhibitors AEW541 and erlotinib, specific against IGF1R and EGFR, respectively, different CRC cell lines exhibited a reduced survival fraction after RCT, with the highest effect after the simultaneous inhibition of IGF1R/EGFR. *In vivo,* xenograft mice simultaneously treated with low dose AEW541/erlotinib plus RCT revealed a significant reduction in tumour volume and weight compared with the tumours of mice treated with either AEW541 or erlotinib alone. *In vitro*, the combined inhibition of IGF1R/EGFR resulted in a stronger reduction of downstream signalling, an increase in DNA double strand breaks (DSBs), apoptosis and mitotic catastrophe after RCT depending on the cell line. Moreover, the existence of IGF1R/EGFR heterodimers in CRC cells and human rectal cancer samples was proven. The heterodimerisation of these RTKs was dependent on the presence of both ligands, IGF-1 and EGF, and functional receptors. In conclusion, these results demonstrate that the strategy of targeting both IGF1R and EGFR, in addition to basic RCT, could be of intriguing importance in CRC therapy.

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, with greater than 1.4 million new cases in the year 2012 [1]. Although combinations of surgery, radiotherapy and chemotherapy (CT) are used, innovative strategies are needed to improve the therapeutic outcome of the patients, especially for the advanced stages of CRC [2]. Therefore, the development of targeted therapies to selectively block molecular pathways that drive CRC is the focus of intensive research [3]. Small molecule inhibitors specific for receptor tyrosine kinases (RTKs) have demonstrated promising effects [4–7]. Several studies indicate that the EGFR is mutated, enhanced and/or constitutively expressed in different tumour entities [5,8,9]. In CRC, the EGFR is overexpressed in up to 82% of patients [10]. The inhibition of EGFR signalling significantly inhibits tumour growth in numerous preclinical models, including colon cancer models [11]. On the basis of these findings, therapeutic strategies, in which the EGFR is inhibited by small molecule inhibitors, such as erlotinib, gefitinib and lapatinib, or inhibitory monoclonal antibodies, such as cetuximab and panitumumab, have been used as clinical therapies in a variety of human malignancies [12–15]. However, responses to targeted therapies are often short-

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lived as tumours acquire resistance, which is associated with signalling, i.e., *via* the IGF1R in the case of EGFR blocking [16–18]. Scartozzi et al. (2010) demonstrated that IGF-1 is a possible predictive factor for the resistance to anti-EGFR monoclonal antibodies in *Kirstein rat sarcoma viral oncogene homolog (KRAS)* wild type CRC patients [19]. Several studies have shown that IGF1R is frequently overexpressed in CRC patients [20–23]. The IGF1R system plays a critical role in the regulation of cell growth and malignant transformation *via* the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways [22,24]. Furthermore, interactions of IGF1R with other RTKs have been reported. In non-small cell lung cancer (NSCLC), erlotinib induces IGF1R/EGFR heterodimerisation on the cell membrane [25]. In MCF7 breast cancer cells, IGF1R interacts with erb-b2 receptor tyrosine kinase 2 (ERBB2) [26,27]. Nahta et al. (2005, 2006) demonstrated that the IGF1R/ERBB2 heterodimer contributes to trastuzumab resistance in breast cancer cells by the activation of multiple receptor pathways, including the IGF1R pathway [27,28]. *In vitro*, the dual silencing of IGF1R/EGFR is associated with decreased proliferation and enhanced apoptosis in CRC cell lines, and signalling crosstalk of IGF1R/EGFR was also observed [6]. However, the molecular basis for the observed positive effects of IGF1R blockade during EGFR-based targeted therapy strategies is not well understood. In addition, the treatment of rectal cancer includes a neoadjuvant 5-FU-based RCT. Therefore, in the current work, we analysed the effect of the simultaneous inhibition of IGF1R/EGFR in combination with RCT on CRC cell growth *in vitro*, as well as on tumour growth in a xenograft mouse model. In addition, we focused on molecular mechanisms underlying the effectiveness of this combinational treatment.

Materials and methods

Details on antibodies, cell lines and drugs, colony formation assay, protein extraction and western blotting, co-immunoprecipitation, immunofluorescence staining, cell-based ELISA, RNA isolation, Reverse Transcription and Quantitative Reverse Transcription-PCR are described in Supplemental Material and Methods.

Proximity ligation assay

Briefly, 2×10^2 cells were plated on culture slides (BD Biosciences) for 24 h. After serum starvation overnight, cells were stimulated with 10 nM IGF-1, 10 ng/ml EGF or 10 nM IGF-1/10 ng/ml EGF, separately, with or without 1 μM AEW541 or 1 μg/ml erlotinib. Cells were fixed in 3.7% formaldehyde for 20 min at RT.

Six human rectal cancer samples from patients treated at the Department of General, Visceral and Paediatric Surgery at the UMG, Germany, which were collected within the clinical research unit KFO 179 upon approval by the ethics committee of UMG (approval number 9/8/08), were used to perform the PLA. Samples were deparaffinised in xylene (Carl Roth), rehydrated through graded ethanol at RT and incubated in citrate buffer (pH 6) for 1 h.

Cells and tissue sections were blocked with 3% BSA for 30 min and incubated with anti- IGF1R and -EGFR antibodies at 4 °C overnight. PLA was performed according to the manufacturer's instructions. Imaging was performed using the confocal LSM (Olympus). For the quantification of heterodimers, the software AlphaView-FluorChem Q SA Version 3.2.2 (Cell Biosciences) was used.

ApoTox-Glo™ Triplex assay

Cell viability, cytotoxicity and caspase activation were evaluated using the ApoTox-Glo™ Triplex assay (Promega, Fitchburg, MA, USA) according to the manufacturer's instructions. Cells were treated with inhibitors and with or without RCT as described above. After 24 h, fluorescence and luminescence were measured with the microplate reader Synergy Mx (BioTek).

Xenograft mouse models and drug treatment

Xenograft experiments were in accordance with the requirements of the German Animal Welfare Act (reference number: G11/0469). Approximately 2×10^6 cells in the logarithmic phase of growth plus 0.5 mg/ml Matrigel were subcutaneously injected into each flank of 5-week-old CD1-Foxn1NU mice (~20 g). At a mean tumour volume of 250 mm^3, mice were randomised into either a treatment group of AEW541 (20 mg/kg; dissolved in 25 mM L(+)-Tartaric acid (Sigma-Aldrich), erlotinib (20 mg/kg; dissolved in 0.2% (w/v) carboxymethyl cellulose with 0.1% Tween 80), AEW541/erlotinib or a control group, with approximately 5 mice per group. Mice were treated with 5-FU-based RCT (dissolved in PBS) (50 mg/kg 5-FU intraperitoneal, followed by 1.8 Gy radiation per day) (Supplemental Material and Methods) plus inhibitors or DMSO (orally applied on 5 consecutive days for 2 weeks). Tumour volume was measured daily using a calliper. If one tumour reached a size of approximately 1500 mm^3, all tumours were dissected from the mice, weighed and measured. Samples for RNA and protein isolation were stored at −20 °C.

Statistical analysis

All *in vitro* studies were performed in two to six independent experiments in replicates as indicated in the figure legend. Means ± s.d. were indicated relative to the control. Student's *t*-test for paired values was used (GraphPad Software Inc., La Jolla, CA, USA). *P*-values < 0.05 were considered statistically significant.

Results

Growth inhibitory effect of the simultaneous inhibition of IGF1R/EGFR plus RCT in vitro

To analyse the effect of targeted therapy plus RCT, colony formation assays (CFAs) were performed with the CRC cell lines SW837, DLD-1 and Caco-2 (Fig. 1A–C). The results were analysed with respect to sensitisation (Fig. 1D–F) and therapy success (Fig. 1G–I). Furthermore, the effectiveness of the inhibitors was calculated using the dose reduction factor (DRF) (Table 1).

In SW837 cells, the single inhibition of either IGF1R or EGFR resulted in sensitisation towards RCT, which was even stronger after the simultaneous inhibition of IGF1R/EGFR (Fig. 1D). This effect was also observed due to an increasing DRF (Table 1). DLD-1 cells exhibited no effect on the survival fraction after the single inhibition of either IGF1R or EGFR. However, the simultaneous inhibition of IGF1R/EGFR resulted in a significant radiation dose-dependent decrease of the survival fraction (Fig. 1E), which is also reflected in the increased DRF (Table 1). In CaCo-2 cells, RTK inhibition did not reveal any sensitisation effect on RCT (Fig. 1F).

The therapy effect of RCT plus inhibitor treatment was more pronounced. In SW837 cells, the inhibition of either IGF1R or EGFR resulted in a reduction of cell survival, which was even enhanced by the simultaneous inhibition of IGF1R/EGFR, leading to a DRF greater than its maximum of 3 (Fig. 1G). DLD-1 cells exhibited no difference in cell survival after the single inhibition of either IGF1R or EGFR. In contrast, the simultaneous inhibition of both IGF1R/EGFR resulted in a clear reduction of the survival fraction (Fig. 1H). However, CaCo-2 cells revealed a reduction of the survival fraction after the single inhibition of either IGF1R or EGFR. The simultaneous inhibition of IGF1R/EGFR even resulted in a complete loss of survival (Fig. 1I).

Simultaneous inhibition of IGF1R/EGFR enhances the effect of RCT in vivo

To assess whether the simultaneous inhibition of IGF1R/EGFR increases the efficacy of RCT *in vivo*, SW837 and DLD-1 cells were implanted subcutaneously into nude mice. Mice were grouped randomly and treated with RCT and low concentrations of AEW541 (20 mg/kg), erlotinib (20 mg/kg) or AEW541/erlotinib (both 20 mg/kg) as depicted in Fig. 2A and Supplementary Figure S2A. Mice did not exhibit any treatment-related toxicity (Supplementary Figure S1).

RCT alone resulted in a clear reduction of tumour growth in the control-treated SW837 xenograft mice. The treatment with low concentrations of either AEW541 or erlotinib reduced tumour growth more effectively compared with control-treated mice. Moreover, an increased reduction in tumour growth could be observed in mice treated simultaneously with AEW541/erlotinib (Fig. 2B). The experiment was terminated after reaching abort criterions, and tumours were measured. The SW837 xenograft mice treated with either AEW541 or erlotinib exhibited a reduction in