Cetuximab promotes anticancer drug toxicity in rhabdomyosarcomas with EGFR amplification *in vitro*

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Abstract. Overexpression of human epidermal growth factor receptor (EGFR) has been detected in various tumors and is associated with poor outcomes. Combination treatment regimens with EGFR-targeting and cytotoxic agents are a potential therapeutic option for rhabdomyosarcoma (RMS) with EGFR amplification. We investigated the effects of combination treatment with actinomycin D and the EGFR-targeting agent cetuximab in 4 RMS cell lines. All 4 RMS cell lines expressed wild-type K-ras, and 2 of the 4 overexpressed EGFR, as determined by flow cytometry, real-time PCR and direct sequencing. Combination of cetuximab and actinomycin D was highly effective, synergistically inhibiting cell growth with a combination index of less than 1. Moreover, combination treatment with these two reagents increased the rate of apoptosis in EGFR-positive cells. Cetuximab has antitumor activity in EGFR-amplified RMS cells when combined with antitumor reagents, indicating that cetuximab is a potential therapeutic reagent for RMS with EGFR amplification.

Introduction

Rhabdomyosarcoma (RMS) is the most common pediatric soft tissue sarcoma. RMS comprises 7-8% of all solid malignant tumors in children and represents approximately two-thirds of all infant sarcomas diagnosed (1). There are 2 distinct histopathological subtypes of this malignancy, embryonal RMS (ERMS) and alveolar RMS (ARMS) (2). In contrast to ERMS, ARMS is characterized by specific translocations, i.e., t(2;13) (q35;q14) in 55% of cases and t(1;13) (p36;q14) in 22% of cases (1). Current treatment options include chemotherapy, complete surgical resection and radiotherapy (3). However, the prognosis for patients with advanced-stage RMS is quite poor (4). The main problems with clinical treatments include metastatic invasion, local tumor recurrence and multidrug resistance. Therefore, more specific, effective and less toxic therapies are required.

Numerous novel anticancer agents are currently in early phase clinical trials. Of these, immunotherapy with specific monoclonal antibodies (mAbs) seems to be a promising approach (5). Alemtuzumab, ibritumomab, rituximab and tositumomab are mAbs already approved for the targeted treatment of white blood cells in leukemia (US Food and Drug Administration). Depending on the level of vascularization, solid tumors may be effectively targeted by bevacizumab, which inhibits vascular endothelial growth factor-A (5).

Identification of the epidermal growth factor receptor (EGFR) as an oncogene has led to the development and approval of panitumumab for the treatment of metastatic colorectal cancer and trastuzumab in breast cancer therapy (5). Cetuximab, a widely used anti-EGFR antibody, consists of a chimeric mouse-human mAb directed against the extracellular domain of EGFR. Cetuximab has been shown to be particularly effective against colorectal cancer and head and neck cancer (6-9) and works by blocking EGFR, leading to inhibition of cell cycle progression (10,11), angiogenesis, invasion and metastasis (12). Treatment with mAbs increases and activates pro-apoptotic molecules in tumor cells (11,13) and enhances cytotoxicity of topotecan (14). Moreover, cetuximab is able to induce antibody-dependent cell cytotoxicity (ADCC) (15-18) and is therefore suitable for immunotherapeutic use. Potential targets for immunotherapy in RMS are not known. The expression of EGFR has been demonstrated in RMS cell lines and tumors. Moreover, previous studies have shown that EGFR expression is a marker for ERMS, with high sensitivity and specificity.

In the present study, we described the distribution of EGFR in human RMS and evaluated the therapeutic potential of cetuximab in RMS patients exhibiting overexpression of EGFR, investigating whether cetuximab affects EGFR-

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dependent apoptosis and enhances the antitumor activity of currently used chemotherapeutic agents in RMS.

Materials and methods

Cell culture and reagents. The ERMS cell lines RD (ATCC, Manassas, VA, USA), RMS-YM and KYM-1 and the ARMS cell line Rh30 (DSMZ, Braunschweig, Germany) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Biochrom, Berlin, Germany) in a humidified atmosphere containing 5% CO_2 at 37˚C. All cells were mycoplasma negative. Cetuximab (Erbitux, Merck, Lyon, France) was obtained from Bristol-Myers Squibb Co. Actinomycin D (Cosmegen; Merck & Co., Inc., Whitehouse Station, NJ, USA) was obtained from Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan).

Flow cytometric analysis. Trypsinized cells were incubated for 30 min in FACS buffer (PBS with 2% FBS, 2 mM EDTA, 0.005% NaN_3; all reagents were from Sigma-Aldrich, Munich, Germany) containing 10 μg/ml cetuximab (Merck, Darmstadt, Germany). Excess antibodies were washed out with FACS buffer, and cells were labeled with FITC-conjugated goat anti-human IgG (Chemicon, Hofheim, Germany). Data were acquired with a FACSCalibur machine (Becton-Dickinson, Heidelberg, Germany) and analyzed by FlowJo software (Tomy Digital Biology, Co., Ltd., Tokyo, Japan). Controls were acquired using rituximab (Roche, Mannheim, Germany) or by omitting cetuximab. To examine the expression of the differentiation and epithelium marker EGFR in RMS cells, the cells were washed and incubated with mouse monoclonal antibodies targeting EGFR-PE (Becton-Dickinson) for 30 min at 4˚C. Cells were then washed and counterstained with 1 μg/ml propidium iodide to label the dead cells.

K-ras mutation assay. DNA was purified from RMS cells using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Sample DNA was added to 8 separate reactions. These reaction mixes contained a single primer set specific for either the wild-type sequence or 1 of 7 mutations in codons 12 and 13. Direct sequencing was conducted using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI Prism 310 DNA Analyzer automated sequencer (Applied Biosystems).

Cell viability assay. Cells were plated in 96-well microplates and cultured for 12 h before exposure to various concentrations of drugs. Cell viability was quantified using the WST-8 assay, determined colorimetrically by measuring the optical density (OD) at a wavelength of 450 nm using a Rainbow Sunrise (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The concentration resulting in 50% growth inhibition (IC_{50}) was calculated for each treatment condition. Data were analyzed to determine the combination index (CI), a well-established index of the interaction between 2 drugs. CI values of <1, 1, and >1 indicate synergistic, additive and antagonistic effects, respectively.

Determination of combination effects. The effects of actinomycin D and cetuximab on growth inhibition were determined as described by Chou and Talalay (19). Briefly, the log (fa/fu) was plotted against the concentration for each compound, alone or in combination, where fa was the fraction affected and fu was the fraction unaffected (1-fa) of cells at each concentration. A CI value <1 represented synergism between actinomycin D and cetuximab, while values equal to or greater than 1 represented additive and antagonistic effects, respectively. The CI was calculated using the Chou-Talalay method in relation to the fraction of cells affected.

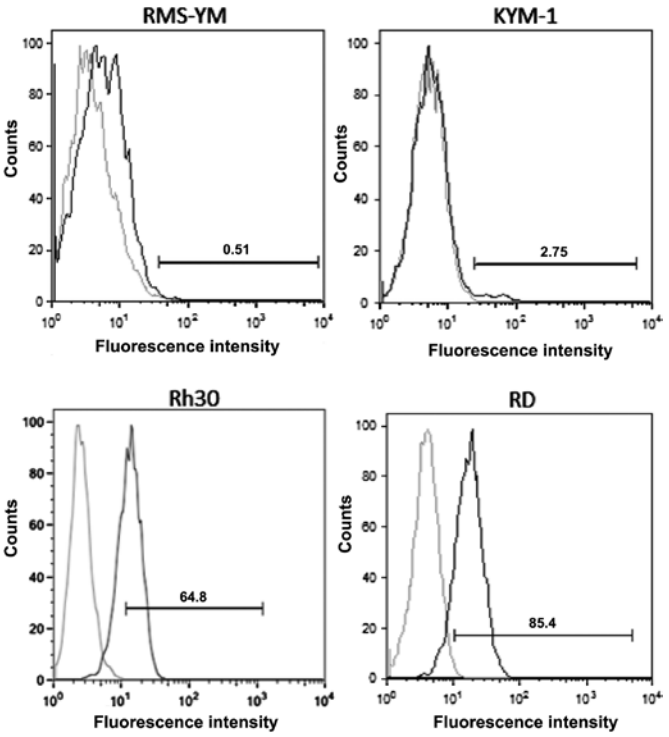

Figure 1. Epidermal growth factor receptor (EGFR) expression analysis in RMS cell lines. High expression of EGFR was detected in RD and Rh30 cells by flow cytometry (black line). The isotype control is also plotted on each panel (gray line).

Analysis of apoptosis by flow cytometry. Cell death was determined through Annexin V-FITC/propidium iodide staining using the TACS Annexin V-FITC Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Following incubation, cells were processed as indicated by the manufacturer and analyzed using FITC and propidium iodide detectors in a FACSCalibur flow cytometer (Becton-Dickinson). Data were analyzed in FlowJo software (Tomy Digital Biology).

RNA isolation and real-time PCR. Total RNA was extracted from untreated cells using the RNeasy Micro kit (Qiagen), and cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis kit (Roche) according to the manufacturer's instructions. Real-time reverse transcription-PCR was carried out in an ABI PRISM 7300 Real-time PCR system (Applied Biosystems). TaqMan gene expression assay primers and probe mixes were used for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and EGFR (assay IDs Hs99999905_m1 and Hs01076078_m1, respectively; Applied Biosystems). GAPDH was detected using TaqMan primers and probes and was used

Table I. Percentage of EGFR-positive cells in the 4 RMS cell lines.

Cell line	Means ± SD
RD	70.0±0.91%
Rh30	65.0±0.78%
KYM-1	0.493±0.066%
RMS-YM	15.9±0.32%

Table II. The IC_{50} values of cetuximab in the 4 RMS cell lines.

Cell line	IC_{50} nM
RD	5333
Rh30	4697
KYM-1	6989
RMS-YM	9119

Table III. Combination index values determined for cetuximab-actinomycin D combinations for the 4 RMS cell lines.

Cell line	Combination index
RD	0.774
Rh30	0.789
KYM-1	1.265
RMS-YM	1.072

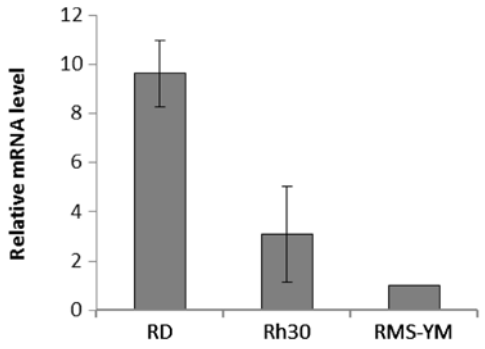

Figure 2. Expression of the EGFR gene as measured by real-time quantitative PCR. The results are shown as fold induction relative to RMS-YM cells (EGFR was not detected in KYM-1 cells).

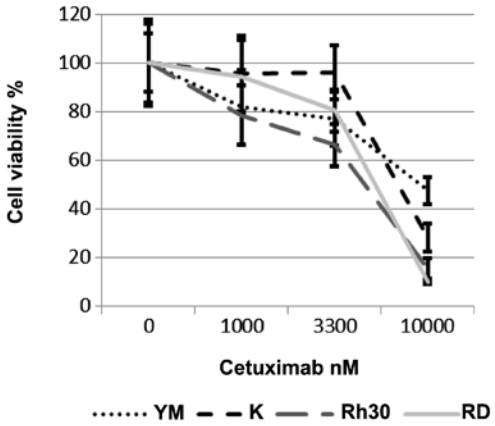

Figure 3. RMS cells were incubated with the indicated cetuximab concentrations for 72 h, and cell viability was measured by WST-8 assay.

as the control gene. The thermal cycling reaction included incubation at 95˚C for 10 min and 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. Relative target mRNA expression was determined using the ΔΔCt method (value obtained by subtracting the Ct value of GAPDH mRNA from the Ct value of the target mRNA). Data were calculated as the ratio of target mRNA to GAPDH mRNA using the $2^{-\Delta\Delta Ct}$ method (20).

Statistical analysis. Determination of the statistical significance of differences between the gene expression analysis groups was carried out using the Student's t-tests in GraphPad Prism 4.00 software (GraphPad Software Inc., La Jolla, CA, USA). All numeric data are expressed as the means ± SD. P-values <0.05 were considered to indicate statistically significant differences.

Results

Expression of EGFR and mutational status of K-ras. The RD and Rh30 cell lines had a large number of EGFR-positive cells, whereas the KYM-1 and RMS-YM cell lines had a small number of EGFR-positive cells (Fig. 1, Table I). Real-time PCR analyses showed that EGFR was overexpressed by 9.62±1.36- and 3.09±1.93-fold in RD and Rh30 cells, respectively, compared with RMS-YM cells; EGFR was not detected in KYM-1 cells in this assay (Fig. 2). Collectively, these data suggest that EGFR is predominantly expressed in RD and Rh30 cells. Sequencing of full-length cDNAs revealed no mutations in codons 12 and 13 of K-ras, suggesting that all 4 RMS cell lines expressed wild-type K-ras (data not shown).

Cetuximab inhibits cell growth in RMS cell lines. Next, we examined the effects of cetuximab on the growth of RMS cells. Cetuximab dose-dependently inhibited the growth of all 4 RMS cell lines, regardless of their EGFR-expression status (Fig. 3). The IC_{50} values of cetuximab in these 4 cell lines were 4.7 μM (Rh30), 5.3 μM (RD), 9.1 μM (RMS-YM) and 7.0 μM (KYM-1) (Table II). IC_{50} value were lower in EGFR-positive cells.

Cetuximab enhances actinomycin D-dependent cytotoxicity in RMS cell lines expressing high levels of EGFR. Incubation with 2 μmol/l cetuximab alone had only a slight effect on the viability of the 4 RMS cell lines, while combination treatment with cetuximab and actinomycin D enhanced drug-induced cytotoxicity in EGFR-amplified cell lines (Fig. 4). This combination effect was synergistic for cetuximab and actinomycin D in RD and Rh30 cells, with CI values <1.0 for both cell lines. By contrast, the combination effect was antagonistic for cetuximab and actinomycin D in RMS-YM and KYM-1 cells, with CI values >1.0 (Table III). Moreover, combination

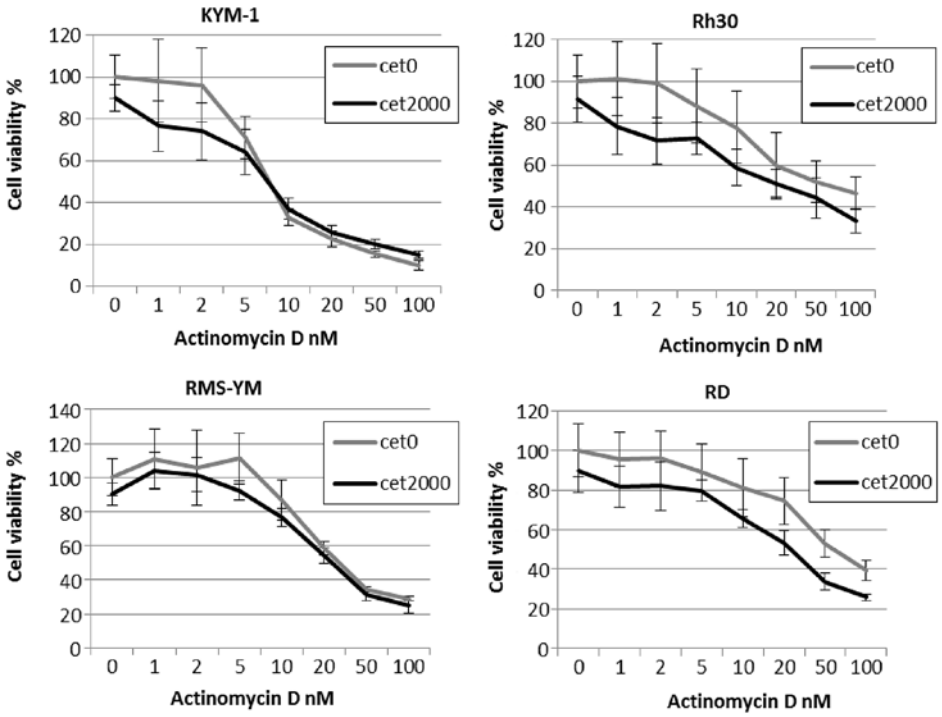

Figure 4. RMS cells were incubated with increasing concentrations of actinomycin D alone (gray line; cet0) or in combination with 2 μM cetuximab (black line; cet2000) for 72 h. Cell viability was assessed by WST-8 assay.

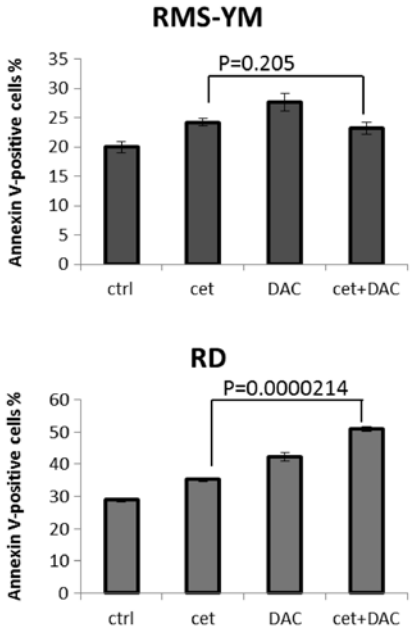

Figure 5. Effects of cetuximab in combination with actinomycin D on RMS cell viability. Viability was assessed by Annexin V-FITC/propidium iodide staining and was measured by flow cytometry. Cells were treated with cetuximab at doses of IC_{50} (RMS-YM; 9119 nM, RD; 5333 nM, respectively), actinomycin D at doses of IC_{50} (RMS-YM, 40 nM; RD, 10 nM, respectively), or combination cetuximab and actinomycin D for 72 h.

treatment with cetuximab and actinomycin D induced apoptosis in EGFR-positive RMS cells. At 72 h after treatment, a greater number of Annexin V-positive cells were detected in RD cells treated with both cetuximab and actinomycin D than in RD cells treated with cetuximab alone (P=0.000214, t-test, statistical significance). The increased apoptosis induced by treatment with these 2 agents was less pronounced in RMS-YM cells than when treated with cetuximab alone (P=0.205, t-test, not significant) (Fig. 5).

Discussion

Although inhibition of EGFR has shown promise as a potential therapeutic treatment in several epithelial malignancies, little is known about its effect on soft tissue tumors. The largest case series reported in the literature demonstrated positive EGFR staining in 60% of human adult soft tissue tumors (n=281) (21). Studies in RMS have shown that expression of EGFR correlates with embryonal subtype (22). It is well established that embryonal subtypes generally behave less aggressively than alveolar subtypes. However, despite advances in the treatment of RMS, the overall 5-year failure-free survival rate does not exceed 80%, even among patients with ERMS or early-stage disease. Novel approaches for the treatment of RMS are required. In the present study, we chose cetuximab, an mAB targeting EGFR that has already been approved for therapeutic applications.

Array-based analysis revealed higher expression of several genes, including BCL2L1 (23), CNR1 (24), CXCR4 (25), MET (26), MYCN (27,28), PDGFR-A (29) and TFAP2(β) (30,31), in ARMS compared with ERMS. Conversely, EGFR (28), HMGA2 (26) and YB-1 (32) were upregulated in ERMS. Of these gene products, CNR1, CXCR4, MET, PDGFR-A and EGFR are localized to the cell membrane and may function as targets for therapeutic antibodies. Our results showed that one ERMS and one ARMS cell line, RD and Rh30, have high EGFR protein expression assessed by flow cytometry, and high expression at the mRNA level by real-time PCR, as previously reported (33).

Mutations in K-ras, which have been reported to occur in colorectal cancer, are responsible for cetuximab resistance in tumor cells. Prior to treatment with cetuximab, K-ras mutations must be monitored (34). However, in a study of RMS tissues, in which a response to blocking antibodies such as cetuximab could be expected, K-ras mutations were detected in only 2 out of 38 ERMS tissues and in no ARMS tissues (n=12) (35). Additionally, RD cells contain only an NRAS mutation (36), and our data showed no mutations in K-ras for RD, RH30, KYM-1 and RMS-YM cells. Although mutations in K-ras are rare in RMS, K-ras mutations should be evaluated prior to treatment for effective treatment with cetuximab.

Actinomycin D is used in current standard treatments for RMS, in combination with vincristine and cyclophosphamide. Herrmann *et al* (33) reported that cell-dependent cytotoxicity of peripheral blood mononuclear cells to RD and Rh30 was enhanced specifically by cetuximab. Herein, we evaluated the treatment effects of cetuximab alone as well as the combination with cetuximab and antitumor reagent. A low concentration of cetuximab in combination with actinomycin D had an enhanced antitumor effect. The combination with cetuximab and actinomycin D was synergistic in inhibiting cell growth, and inducing cell apoptosis, with a CI of <1 in EGFR amplified cells, RD and Rh30. By contrast, the combination was antagonistic in RMS cells without EGFR amplified, with a CI of >1. Apoptotic cells without EGFR amplified RMS, treated with cetuximab and actinomycin D, were fewer than those treated with actinomycin D alone (Fig. 5), suggesting that the combination of cetuximab and standard chemotherapy including actinomycin D may be a promising therapeutic strategy for patients with EGFR amplified RMS, but not for patients without EGFR amplified RMS. Previous studies reported that activation of EGFR leads to downstream signaling that activates mitogenic and survival pathways, such as the MAPK and Pi3-K/AKT pathways (37). Inhibition of these pathways by an EGFR antagonist, such as cetuximab, can lead to induction of apoptosis and anti-proliferative effects (38). These results suggest that combination therapy may block the signaling pathways downstream of EGFR.

In summary, we have shown that combination of cetuximab and actinomycin D resulted in antitumor activity against human RMS cell lines expressing high levels of EGFR, suggesting that EGFR antagonists may be promising therapeutic interventions for the treatment of RMS. Further animal studies and clinical trials are required to evaluate the safety of EGFR antagonists.

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