Cetuximab Sensitivity Associated with Oxaliplatin Resistance in Colorectal Cancer

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Abstract. *Background: Clinical studies have suggested that the epidermal growth factor receptor (EGFR)-inhibiting antibody cetuximab may have better effect in the third-line treatment of metastatic colorectal cancer, after failure of standard chemotherapy including oxaliplatin, compared to using it up-front in the first line. The reason behind this suggestion is unclear. Materials and Methods: The effect of cetuximab on cell growth was investigated in five isogenic colon cancer cell lines with increasing level of acquired oxaliplatin resistance. The expression of EGFR and the activity of down-stream signalling molecules were measured by western blot analyses. Results: A marked increase in sensitivity to cetuximab, accompanied by an up-regulation of EGFR, was observed in the oxaliplatin-resistant cell lines. Conclusion: The connection between oxaliplatin resistance and cetuximab sensitivity has not been previously reported in the literature. Such a connection could be of clinical importance and constitutes a substantial an argument for saving cetuximab until later treatment lines, when the tumours have become chemotherapy resistant.*

The oncological armoury for modern management of colorectal cancer (CRC) includes both classic cytostatics and targeted substances. One of the most frequently used chemotherapeutic agents for this disease is oxaliplatin. In recent years, drugs targeting the epidermal growth factor receptor (EGFR) have shown efficacy in CRC. Cetuximab is an antibody directed against EGFR.

Randomised studies regarding the addition of cetuximab to oxaliplatin-based chemotherapy in the first-line treatment of metastatic CRC have yielded conflicting results. One study showed a significant increase in progression-free survival (hazard ratio=0.57, median 7.2 *vs*. 7.9 months) in patients whose tumours were v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) wild-type (*KRAS*wt) (1), whereas two recent studies have failed to show any benefit from adding cetuximab to oxaliplatin-based first-line chemotherapy (2, 3).

In the third line of therapy, after failure of standard chemotherapy, a randomised study revealed a highly significant increase in median overall survival (OS) with cetuximab compared with best supportive care, from 4.8 months to 9.5 months, in patients with *KRAS*wt tumours (4). These data raise the question whether cetuximab is more effective in later treatment lines, and whether this could be a consequence of resistance development to chemotherapeutic drugs. To test this hypothesis, we investigated the sensitivity of colon cancer cell lines, with acquired resistance to oxaliplatin, to cetuximab.

Materials and Methods

We used a human colon cancer cell line, S1 (derived from the S1 clone of LS-174T colon carcinoma cells (5), which was heterozygous for the Gly12Asp *KRAS* mutation), and the increasingly oxaliplatin-resistant subclones S1-oxa2, S1-oxa4, S1-oxa5 and S1-oxa6 which were developed with 2, 4, 5 and 6 μmol/l oxaliplatin, respectively (6). The cell lines were cultured as described earlier (6).

To measure the growth inhibiting effect of cetuximab, the cell lines were seeded at a density of 1500 cells per well in 96-well plates. After two days, the medium was aspirated and new medium with a four fold dilution series of cetuximab (from 200 nmol/l to 3.1 pmol/l), or control medium without antibody was added. The cells were left to grow for five days, after which the cell numbers were determined using the sulphorhodamine B (SRB) assay (7).

The expression of EGFR and the activation status of v-akt murine thymoma viral oncogene homolog (AKT), extracellular-signal-regulated kinases 1 and 2 (ERK1/2) and signal transducer and activator of transcription 3 (STAT3) were analyzed by western blotting. The cells were grown under ordinary growth conditions to 90% confluency in 100-mm dishes and then lysed in 750 μl ice-cold RIPA buffer [1% Triton X-100, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate in 150 mmol/l NaCl, 2 mmol/l Na_3VO_4, 1 mmol/l NaF, 20 mmol/l $Na_4P_2O_7$, Complete Protease Inhibitor (Roche Applied Science, Basel, Switzerland) and 50 mmol/l Tris-HCl, pH 7.4]. The protein concentration was

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determined by micro BCA protein assay (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a standard. Equal protein amounts were separated by 4-12% NuPAGE, Bis-Tris electrophoresis (Invitrogen, Carlsbad, CA, USA) and then transferred to polyvinylidene fluoride (PVDF) membranes. The primary antibodies used were anti-EGFR (cat. no. 2232), anti-phospho-p44/p42 mitogen-activated protein kinase (MAPK) (Thr202/Tyr204) (cat. no. 9101), anti-phospho-STAT3 (Tyr705) (cat. no. 9131), and anti-phospho-AKT (Ser473) (cat. no. 9271) all from Cell Signaling Technology (Danvers, MA, USA). Detection was performed with goat anti-rabbit horseradish peroxidase (HRP)-linked antibody (cat. no. 7074; Cell Signaling Technology) in combination with the ECL plus detection kit (GE Healthcare, Waukesha, WI, USA). The staining intensity was determined using a FluorChem FC2 with AlphaView software (Cell Biosciences, Santa Clara, CA, USA) and Coomassie protein staining was used as loading control as previously described (8). Specific expression of each protein species was calculated by dividing the immunostaining intensity with the Coomassie staining intensity.

Results

The effect of cetuximab on cell growth was measured *in vitro* using the SRB assay. The parental S1 cell line was inhibited by cetuximab to a maximum of 20%, consistent with earlier results (7). The least oxaliplatin-resistant cell line, S1-oxa2, displayed an equal sensitivity towards cetuximab but the other three cell lines were inhibited by up to a maximum of around 70% (S1-oxa6) (Figure 1). The experiment was repeated three times with similar results.

To investigate if the oxaliplatin resistance was coupled to increased EGFR expression, which could explain the change in cetuximab sensitivity, cell lysates were subjected to western blotting. The EGFR expression increased proportionally in relation to the level of acquired resistance to oxaliplatin, to a maximum in S1-oxa6, 3.5 times higher than the one of S1 (Figure 2).

EGFR downstream signalling was measured by western blotting. Phosphorylated STAT3 was not detected in any of the cell lines (data not shown). ERK1/2 was phosphorylated to a barely detectable level (Figure 3A) with a tendency for higher phosphorylation in the more oxaliplatin-resistant cell lines (Figure 3B). AKT displayed an inverted bell-shaped activation curve with the lowest phosphorylation level observed in S1-oxa4 and equally high phosphorylation observed in S1 and S1-oxa6 (Figure 3A and B).

Discussion

Two major mechanisms have been suggested for the cytostatic action of cetuximab – direct inhibition of EGFR signalling through ligand binding competition, and activation of the immune system in the so called antibody-dependent cellular cytotoxicity (ADCC). In combination with, for example, tumour uptake issues, this limits the usefulness of elaborate *in vitro* studies to predict clinical effects of the substance. However, cell experiments can be used for the generation of hypotheses that can be further tested in clinical materials.

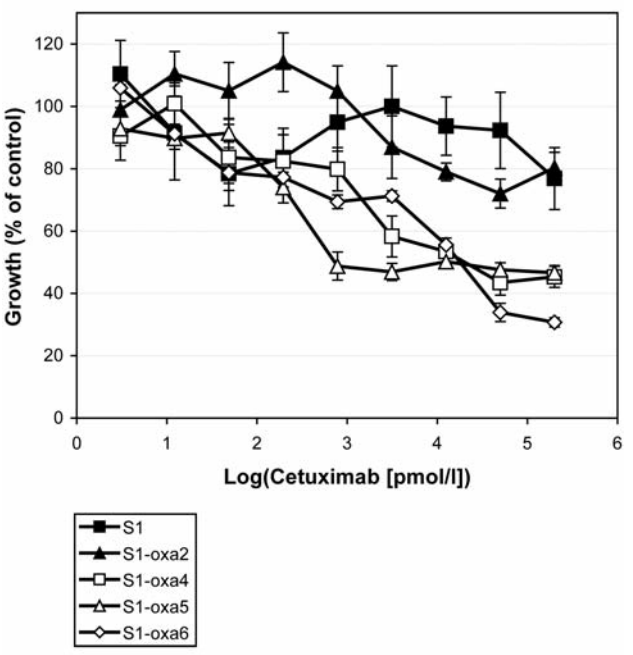

Figure 1. *The colon cancer cell line S1 and its oxaliplatin resistant derivatives S1-oxa2, S1-oxa4, S1-oxa5 and S1-oxa6 were incubated with increasing concentrations of cetuximab for five days. Cell numbers were measured with the sulphorhodamine B assay, ±SEM (N=6).*

In the present study, we were interested in the possibility of cells developing an increased sensitivity to cetuximab when acquiring resistance to oxaliplatin. To test this suggestion, we measured the cetuximab sensitivity in a set of five colon cancer cell lines, previously made resistant to oxaliplatin, and found a marked increase in the sensitivity to cetuximab with increasing oxaliplatin resistance. This has, to our knowledge, not been reported previously. We also found that increasing oxaliplatin resistance was accompanied by an increase in EGFR expression.

These results suggest that EGFR signalling could be involved in acquired resistance to oxaliplatin. A connection between resistance to platinum drugs and EGFR expression has been described in breast cancer cell lines (9). It has also been shown that EGFR signalling can increase DNA repair through up-regulation of the DNA repair proteins, X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) and excision repair cross-complementing rodent repair deficiency complementation group 1 (ERCC1) (10). In addition, several reports have indicated that EGFR expression can be up-regulated by exposure to platinum drugs (11, 12). In line with this, it was shown that cetuximab increased the effect of oxaliplatin in cell lines with acquired

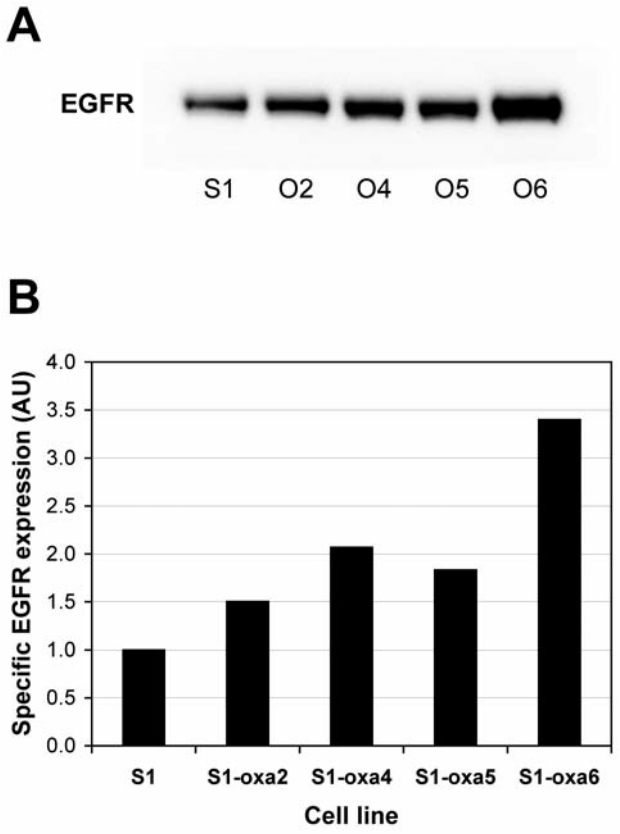

Figure 2. *Epidermal growth factor receptor (EGFR) expression. Cell lysates from S1 and the oxaliplatin resistant derivatives were analysed for EGFR protein expression by western blotting (A). The densities of the EGFR bands were measured in AlphaView software and are related to the density of protein staining in each lane (B). The western blot was repeated four times and a representative experiment is shown. The results were similar, showing approximately 3.5 times higher EGFR expression in S1-oxa6 cells as compared to S1 cells.*

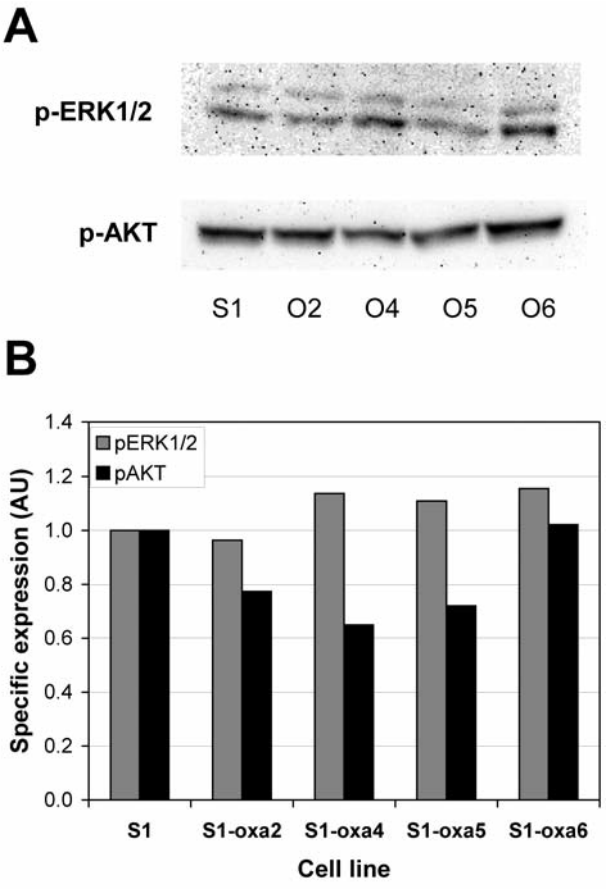

Figure 3. *Epidermal growth factor receptor (EGFR) downstream signalling. Cell lysates from S1 and the oxaliplatin resistant derivatives were analysed for phosphorylation of EGFR downstream signalling proteins by western blotting (A). The densities of the bands were measured in AlphaView software and were related to the density of the protein staining in each lane (B). The western blot was repeated three times and a representative experiment is shown.*

oxaliplatin resistance (13). These reports show that resistance to platinum drugs can be affected by changes in EGFR activity. The current results further imply that such changes might affect EGFR-dependent cell proliferation. The oxaliplatin-resistant cell lines were thus more dependent on ligand-induced EGFR signalling for cell growth compared to the parental cell line, as shown by the higher sensitivity to cetuximab inhibition. The increased EGFR expression in the oxaliplatin-resistant cell lines could be important for this change, but a redirection of intracellular signalling would also be necessary to explain how the activating *KRAS* mutation could be overridden. An indication that EGFR downstream signalling might be affected were the alterations seen in basal activity of AKT and ERK1/2 in the oxaliplatin-resistant cell lines. The latter is in line with the previously described ERK-dependent EGFR activation of ERCC1 and XRCC1 in prostate carcinoma cell lines (10).

The fact that the parental cell line carried an activating *KRAS* mutation complicates the interpretation of the results. Most clinical studies in colorectal cancer have shown that tumours harbouring a *KRAS* mutation are resistant to cetuximab treatment. However, in the recently presented Nordic VII trial (13), there was a tendency for improved progression-free survival in patients with *KRAS*-mutated tumours that received cetuximab in addition to oxaliplatin-based chemotherapy, whereas patients with *KRAS*wt tumours had no benefit at all from the addition of cetuximab. The reason behind these findings is unclear, but it indicates that there are still issues regarding *KRAS* status and effects of EGFR inhibitors that need to be clarified.

It should also be remembered that clinical treatment with cetuximab is prolonged over several months, while in the current study, the cells were treated with a single dose and assayed after five days. Thus, any long-term effects of the

activating *KRAS* mutation were not taken into account in this experimental set-up, but would probably be of importance for the outcome in a clinical situation. Nevertheless, the results show that resistance to oxaliplatin can be coupled to an increase in cetuximab sensitivity at the cellular level and also in an increase of EGFR expression. Thus, both direct effects on cell proliferation and an increased ADCC response might potentially increase the effect of cetuximab treatment. Considering the complexity of EGFR signalling and the multitude of mutations inferring resistance to cetuximab, it is reasonable to believe that this will not apply equally to all cell lines or tumours. However, our results could constitute an explanation for the positive clinical effects of cetuximab often seen when used in the second or third line after failure of platinum-based therapies. This would, in turn, be an argument for saving the use of cetuximab until later treatment lines, rather than using this drug up-front regarding management of patients with metastatic CRC.

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