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Beschreibung:

Association of amphiregulin with the cetuximab sensitivity of gastric cancer cell lines

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Abstract. The therapeutic activity of the epidermal growth factor receptor (EGFR)-directed monoclonal antibody cetuximab in gastric cancer is currently being investigated in clinical studies. Reliable biomarkers for the identification of patients who are likely to benefit from this treatment are not available. In this study, we assessed the activity of cetuximab in five gastric cancer cell lines (AGS, AZ521, Hs746T, LMSU and MKN1). The viability of two of these cell lines, AZ521 and MKN1, was significantly reduced by cetuximab treatment. High expression and secretion levels of the EGFR-binding ligand, amphiregulin (AREG), were associated with cetuximab responsiveness. MET activation and mutations in *Kirsten-Ras* gene (*KRAS*) were associated with cetuximab resistance. By introducing a hierarchy between these markers, we established a model that facilitated the correct classification of all five gastric cancer cell lines as cetuximab responsive or non-responsive. The highest priority was allocated to activating *KRAS* mutations, followed by MET activation and finally by the levels of secreted AREG. In order to validate these results, we used three additional human gastric cancer cell lines (KATOIII, MKN28 and MKN45). In conclusion, we propose that our model allows the response of gastric cancer cell lines to cetuximab treatment to be predicted.

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Abbreviations: AREG, amphiregulin; CRC, colorectal cancer; DMEM, Dulbecco's modified Eagle's medium; ECACC, European Collection of Cell Cultures; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; EREG, epiregulin; IU, international units; *KRAS*, *Kirsten-Ras* gene; MEM, Minimum Essential Medium Eagle; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; rpm, rounds per minute; RTK, receptor tyrosine kinase; SCCHN, squamous cell carcinoma of the head and neck; SD, standard deviation

Key words: epidermal growth factor receptor, cetuximab, gastric cancer, amphiregulin

Introduction

With approximately 738,000 deaths per year and an overall five-year survival rate below 30%, gastric cancer is the second most common cause of cancer mortality worldwide (1-3). This high level of mortality is due to the fact that most gastric cancer patients are diagnosed with locally advanced or metastatic disease. Therefore, the treatment options are limited, and new therapeutic approaches are urgently needed.

The dysregulation of the expression of the epidermal growth factor receptor (EGFR) is considered an important step in tumorigenesis. As EGFR is overexpressed in approximately 27 to 42% of gastric tumors (4,5), it represents an interesting target for therapeutic intervention. One therapeutic approach is the use of monoclonal antibodies that target EGFR. Cetuximab is one such monoclonal antibody and is approved for the treatment of recurrent colorectal cancer (CRC) and squamous cell carcinoma of the head and neck (SCCHN). Furthermore, a number of phase II studies on gastric cancer patients treated with various combinations of cetuximab with chemotherapy have shown promising results (6-9). A multinational phase III trial of cetuximab plus chemotherapy is on-going.

Experience with CRC and SCCHN therapies has shown that only a subgroup of patients benefits from cetuximab-based therapy. Well-known cetuximab resistance mechanisms include activating mutations in the *Kirsten-Ras* gene (*KRAS*), leading to a constantly activated EGFR pathway (10). Nevertheless, in many cases, the underlying resistance mechanism remains unclear. For optimal patient selection, it is therefore necessary to identify additional markers that are predictive of the cetuximab response.

On the molecular level, cetuximab inhibits EGFR activation by binding to the EGFR ligand binding site with a significantly higher affinity than the members of the family of EGFR-binding ligands (11). This group of EGFR-binding ligands consists of the epidermal growth factor (EGF), amphiregulin (AREG),

epiregulin (EREG), epigen (EPGN), transforming growth factor α (TGFα), betacellulin (BTC) and heparin-binding EGF (HB-EGF) (12-17). Ligand binding results in EGFR dimerization, the activation of its tyrosine kinase domain and subsequent signal transduction via a cascade of EGFR signaling pathway intermediates. Finally, processes that play an important role in tumorigenesis are activated; these processes include cell survival, proliferation and migration. In contrast to ligand binding, cetuximab binding results in EGFR internalization without further activation of the receptor and the downstream signaling pathway (18).

Previously, it was shown that AREG expression and EREG expression are positive predictive markers for the outcome of CRC patients treated with cetuximab in combination with chemotherapy (19,20). Among *KRAS* wild-type tumors, patients with high expression levels of AREG and EREG are highly likely to respond to these therapy regimens. In addition to *KRAS*, AREG and EREG, a predictive role in EGFR-inhibitory therapy has been discussed for EGFR, EGFR signaling intermediates, such as PI3K, PTEN or BRAF, and other EGFR ligands (21). A number of studies have suggested a negative predictive value for other receptor tyrosine kinases (RTKs), such as the hepatocyte growth factor receptor, MET (22-24).

In gastric cancer, the search for predictive markers for the response to cetuximab-based therapies is on-going. As the prevalence of *KRAS* mutations in gastric cancer is low (25), a correlation between *KRAS* mutations and therapy response is difficult to establish. In contrast to CRC, no significant correlation between AREG expression and the response rate was found in gastric cancer patients treated with cetuximab in combination with oxaliplatin, leucovorin and 5-fluorouracil in a recent clinical phase II trial (26).

In the present study, we analyzed the cetuximab responsiveness of several gastric cancer cell lines (AGS, AZ521, Hs746T, LMSU and MKN1) and assessed the predictive value of EGFR and its ligands AREG and EGF alone and in combination with activating *KRAS* mutations and MET activation. We introduced a hierarchy between these markers and established a model that facilitates the correct classification of all five gastric cancer cell lines as cetuximab-responsive or -non-responsive. The model was validated with three other human gastric cancer cell lines, KATOIII, MKN28 and MKN45.

Materials and methods

Cell lines and cultivation conditions. The human gastric cancer cell lines AGS, KATOIII, MKN1, MKN28 and MKN45 were cultured in RPMI-1640 medium (Invitrogen/Gibco, Darmstadt, Germany) supplemented with 10% fetal bovine serum Sera Plus (PAN Biotech, Aidenbach, Germany), 2 mM l-glutamine (Invitrogen/Gibco) and penicillin-streptomycin (PAA Laboratories, Pasching, Austria; 100 international units (IU)/ml, 100 μg/ml) as reported previously (24). LMSU cells were grown in Nutrient Mixture F-10 Ham medium (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), AZ521 cells were cultured in Minimum Essential Medium Eagle (MEM, Sigma-Aldrich Chemie GmbH), and Hs746T cells were grown in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™ I (Invitrogen/Gibco), 4500 mg/l d-glucose and sodium pyruvate; all three media were supplemented with 10% fetal bovine serum Sera Plus and penicillin-streptomycin (100 IU/ml, 100 μg/ml). The cells were grown at 37°C in a humidified 5% CO_2 atmosphere. The absence of mycoplasma was ensured in the conditioned medium after thawing frozen cells. Cell lines were used until passage 30.

Cell line source and cell validation testing. The AGS and KATOIII cells were obtained from the European Collection of Cell Cultures (ECACC), a Health Protection Agency Culture Collection Supplier of authenticated and quality controlled cell lines and nucleic acids (Porton Down, Salisbury, UK; http://www.hpacultures.org.uk/collections/ecacc.jsp). MKN1, AZ521 and LMSU cells were supplied by the Cell Bank RIKEN BioResource Center (Tsukuba, Japan). MKN28 cells were kindly provided by Dr V. Wachek (Medical University of Vienna, Vienna, Austria). MKN45 cells were obtained from Professor M. Ebert (Technische Universität München, Klinikum rechts der Isar, Munich, Germany). Hs746T cells were obtained from the ATCC Cell Biology Collection (LGC Standards GmbH, Wesel, Germany). The cell validation testing was performed as described previously (24). In addition, authentication of the Hs746T cell line was performed by short tandem repeat profiling using the Cell ID™ system (Promega, Mannheim, Germany).

XTT cell proliferation assay. The XTT cell proliferation kit II (Roche Diagnostics, Mannheim, Germany) was used according to the manufacturer's instructions to assess growth inhibitory effects, as described previously (24). A modification of the US National Cancer Institute (NCI) protocol for *in vitro* cancer screens was used to determine cellular sensitivity to cetuximab (27,28). According to the individual doubling times of the different cell lines, cells were plated at densities between $1x10^3$ and $4x10^3$ cells per well in 80 μl of culture medium.

After 24 h of incubation at 37°C and 5% CO_2, cetuximab (Merck, Darmstadt, Germany) was added at concentrations between 0 and 200 μg/ml in 20 μl of culture medium. A cetuximab concentration of 100 μg/ml is comparable to the active drug concentrations achieved in cancer patients (29-31). After 48 h of incubation, 50 μl of XTT labeling mixture was added per well, and after 2 h at 37°C and 5% CO_2, the absorbance of the samples was measured using a microplate reader (Asys Expert Plus, Biochrom, Berlin, Germany).

DNA isolation and sequencing. DNA isolation was performed using a standard protocol. Cells ($1x10^6$) were harvested, washed twice with phosphate-buffered saline (PBS) [centrifugation at 1,000 rounds per minute (rpm), 3 min] and transferred to 1.5 ml reaction tubes. The cell sediment was resuspended in 200 μl of 50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.5 % Tween-20 and 0.2 mg/ml proteinase K. After a 3-h incubation at 55°C, proteinase K was inactivated by boiling for 10 min, and aliquots were used for the mutation analysis.

The sequences of the primers used for the polymerase chain reaction (PCR) were as follows (from 5' to 3'): BRAF forward (F), ACAGTAAAAATAGGTGATTTTGGTCTAG CTACAGA; BRAF reverse (R), CTATGAAAATACTATAG TTGAGACCTTCAATGACTTTC; EGFR exon 18 F, AGGG CTGAGGTGACCCTTGT; EGFR exon 18 R, TCCCCACC AGACCATGAGAG; EGFR exon 19 F, GCACCATCTCACA

ATTGCCAGTTA; EGFR exon 19 R, AAAAGGTGGGCC TGAGGTTCA; EGFR exon 21 F, CCTCACAGCAGGGT CTTCTCTGT; EGFR exon 21 R, TCAGGAAAATGCT GGCTGACCTA; KRAS exon 2 F, GGTGGAGTATTTGATA GTGTATTAACC; KRAS exon 2 R, CCTCTATTGTTGGAT CATATTCG; PIK3CA exon 9 F, TTGCTTTTTCTGTAAATC ATCTGTG; PIK3CA exon 9 R, CTGCTTTATTTATTCCAA TAGGTATG; PIK3CA exon 20 F, TGACATTTGAGCAAA GA CCTG; PIK3CA exon 20 R, CCTATGCAATCGGTCTTTGC.

The *BRAF* hotspot mutation, V600E, was analyzed using allele-specific PCR following an established protocol (32). Positive and negative controls that had been verified by direct sequencing were included in this analysis. The primers for the *EGFR* mutation analysis were described previously (33,34). The *KRAS* mutation analysis was performed as reported previously (7). The *EGFR*, *KRAS* and *PIK3CA* mutation analysis was performed using PCR amplification followed by direct sequencing.

DNA sequencing analysis was performed using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and the products were separated using an automated sequencing system (3130 Genetic Analyzer, Applied Biosystems).

Enzyme-linked immunosorbent assay (ELISA). For the ELISA-based detection of AREG and EGF, DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA) were used according to the manufacturer's instructions. The ligand concentrations were measured in the conditioned medium and in the cellular extract.

For the determination of the AREG and EGF levels in the conditioned medium of the cell cultures, $1x10^6$ cells were seeded into cell culture plates (10 cm in diameter) and cultured in 10 ml of medium. EGF and cetuximab were added 2 h after the cultures were seeded. At the indicated time-points, the conditioned medium was harvested and centrifuged to remove the cell debris (13,000 rpm, 4°C, 10 min). The supernatant was used for subsequent analysis.

For the determination of AREG and EGF levels in the cellular extract, $5x10^5$ cells (AGS, AZ521), $8x10^5$ cells (MKN28) or $1x10^6$ cells (Hs746T, KATOIII, LMSU, MKN1 and MKN45) were seeded as described above and cultured for 48 h. Subsequently, the cells were lysed in 350 μl of lysis buffer (7X lysis buffer: 20 mM Tris-HCl, pH 7.5; 1 mM EDTA; Complete mini protease inhibitor cocktail tablets, Roche Applied Science, concentration according to the manufacturer's instructions) and sonicated (25 sec, amplitude 70%). The cell debris was removed by centrifugation as described above. A volume of 100 μl of total protein (100 μg/ml) was used for each sample.

Western blot analysis. The expression levels of EGFR, phosphorylated EGFR (Y1068) and phosphorylated MET (Y1234/1235) were determined using a standard protocol. For the western blot analysis, cells were seeded at a density of $1x10^6$ cells per 10-cm tissue culture dish. After 48 h, the cells were washed with ice-cold PBS and lysed on ice with 150 μl of L-CAM lysis buffer as described previously (35). Between 10 and 30 μg of total protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (GE Healthcare, Munich, Germany, no. RPN303F).

After 1 h of blocking, the membranes were probed with appropriate primary antibodies overnight at 4°C: anti-EGFR rabbit polyclonal antibody (no. 2232, Cell Signaling Technology, distributed by New England Biolabs, Frankfurt, Germany; dilution 1:2,000), anti-phosphorylated EGFR (pEGFR) rabbit polyclonal antibody directed against tyrosine residue 1068 (no. 44788G, Invitrogen, Karlsruhe, Germany; dilution 1:2,000), anti-phosphorylated MET (pMET) rabbit monoclonal antibody directed against tyrosine residues 1234 and 1235 (no. 3077, Cell Signaling Technology, dilution 1:1,000), anti-α-tubulin mouse monoclonal antibody (no. T6199, Sigma-Aldrich Chemie GmbH; dilution 1:10,000) and anti-β-actin mouse monoclonal antibody (no. A1978, Sigma-Aldrich Chemie GmbH; dilution 1:5,000).

Detection was performed using horseradish peroxidase-conjugated secondary antibodies by enhanced chemiluminescence (Amersham, Braunschweig, Germany). For signal quantification, blots were scanned and densitometrically analyzed using Image J software 1.42q (National Institute of Health, MD, USA).

Flow cytometry analysis. Cells were seeded at densities of $3x10^5$ to $1x10^6$ cells per 10-cm tissue dish and cultured for 72 h. After washing with PBS (without Ca^{2+}/Mg^{2+}), the cells were detached using 1 ml of Versene (Invitrogen/Gibco) for 10-15 min at 37°C. Detached cells were resuspended in ice-cold FACS buffer [1% (w/v) bovine serum albumin (BSA; Sigma-Aldrich Chemie GmbH) in PBS (without Ca^{2+}/Mg^{2+})]. After two additional washing steps [each comprising i) resuspension of the cells in FACS buffer, ii) sedimentation at 300 g (4°C) for 3 min and iii) discarding the supernatant], the cells ($1x10^5$) were incubated with monoclonal antibody directed against EGFR (Ab-1, clone 528, Thermo Fisher Scientific, Ulm, Germany; 2.5 μg/ml) in 50 μl of FACS buffer in 96-well microtiter plates. Additionally, an isotype control antibody [IgG2a, clone PPV-04, Exbio, Prague, Czech Republic, distributed by Biozol (Eching, Germany; 2.5 μg/ml)] was routinely applied. After incubation for 1 h at 4°C in the dark, the cells were washed twice as described above and incubated with 50 μl of secondary antibody solution [Alexa Fluor 647 F(ab)2-fragment (H+L), Invitrogen, 5 μg/ml] for 1 h (4°C) in the dark. Two washing steps were performed to remove unbound secondary antibodies. The cells were then resuspended in 300 μl of FACS buffer and subsequently analyzed using a FACSCanto flow cytometer (BD Bioscience, Heidelberg, Germany). For live-dead discrimination, propidium iodide was added to each sample at a final concentration of 1 μg/ml directly before measurement.

Statistical analysis. The data are presented as the means ± standard deviation (SD). Pairwise comparisons of samples were performed by two-sided Welch's t-tests. In the XTT cell proliferation assay, one sample t-tests were used to test the activity ratio of treated samples to untreated samples against a reference value of 100% which indicates equality of activity. All analyses were performed on an explorative significance level of 0.05 using the statistical software package R (The R Foundation for Statistical Computing, Vienna, Austria). P-values at a significance level of <0.05 are indicated by (*) and <0.01 by (**). A summary of all statistical data is available from the authors upon request.

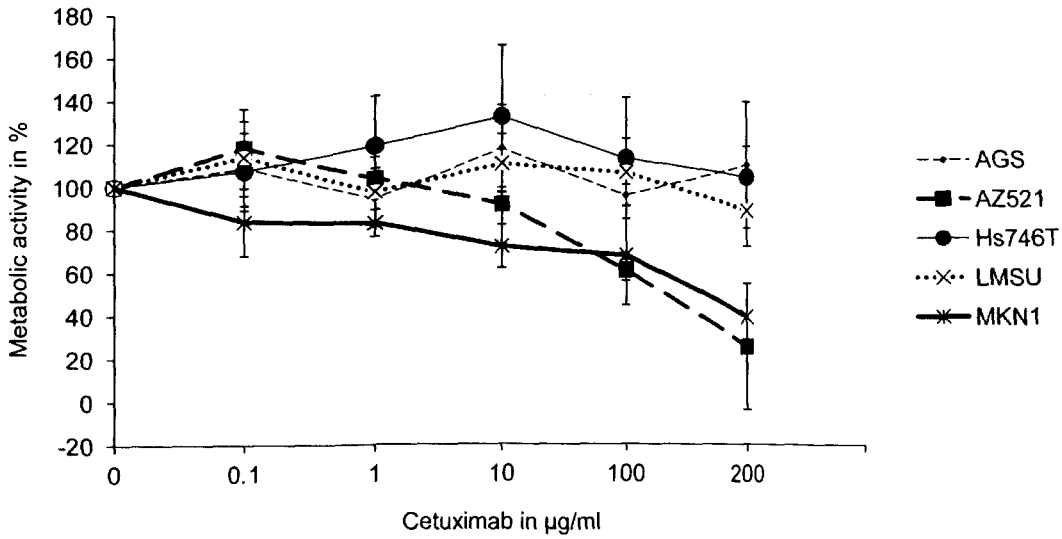

Figure 1. The effect of cetuximab treatment on the metabolic activity of gastric cancer cell lines. The gastric cancer cell lines, AGS, AZ521, Hs746T, LMSU and MKN1, were incubated for 48 h with different concentrations of cetuximab. Subsequently, an XTT assay analysis was performed to evaluate the metabolic activity of the cells. The values were normalized to those of the untreated control. The mean values of at least three independent experiments are shown. The error bars indicate the means ± SD.

Results

Cetuximab responsiveness of gastric cancer cell lines. The EGFR signaling pathway is involved in the regulation of tumor cell proliferation. To determine the cetuximab responsiveness of a panel of five human gastric cancer cell lines (AGS, AZ521, Hs746T, LMSU and MKN1), cells were treated with varying concentrations of the therapeutic antibody (0-200 µg/ml cetuximab). The metabolic activity of the cell lines as a surrogate marker for cell viability was analyzed with the XTT cell viability assay.

The concentration-response curves indicate that the metabolic activities of AZ521 and MKN1 cells were significantly reduced by cetuximab in a concentration-dependent manner (Fig. 1). Significant effects on MKN1 cells were observed at a cetuximab concentration of 1 µg/ml and on AZ521 cells at a cetuximab concentration of 100 µg/ml. The cell lines, AGS, LMSU and Hs746T, were not cetuximab-responsive.

Based on these results, the gastric cancer cell lines, AZ521 and MKN1, were classified as cetuximab-responsive, whereas the other considered cell lines (AGS, Hs746T and LMSU) were regarded as cetuximab-resistant.

Association of cetuximab responsiveness with the AREG and EGF levels. Previously, a correlation between the levels of EGFR ligands and the response to cetuximab-based therapy was demonstrated. The ligand, AREG, was shown to have a high predictive value for the response to EGFR inhibitory therapy in CRC patients (19), whereas a low EGF level was associated with a higher probability to respond to cetuximab plus chemotherapy in gastric cancer patients (26). To clarify the predictive role of EGFR ligands in our gastric cancer model, we determined the amounts of AREG and EGF secreted into the conditioned medium and in the cellular extract by ELISA. Using this approach, it was possible to distinguish between proteolytically released soluble ligands and membrane-bound ligands.

As shown in Fig. 2, AREG was expressed and secreted by several cell lines. AZ521 cells displayed the highest amount of AREG, with a mean concentration of 1,375 pg/ml in the conditioned medium and 1,017 pg/mg in the cellular extract. A high level of AREG was also detected in the MKN1 cells (519 pg/ml in the conditioned medium and 746 pg/mg in the cellular extract). In comparison, only low amounts of AREG were observed for the cell line Hs746T. In these cells, the AREG concentrations were 135 pg/ml in the conditioned medium and 100 pg/mg in the cellular extract, levels 10-fold lower than those in the AZ521 cells. It was not possible to detect significant concentrations of AREG in the conditioned media of the AGS and LMSU cell cultures. Furthermore, analysis of the cellular extract revealed only marginal amounts of the ligand in these two cell lines.

EGF was not found in significant concentrations in the cell extracts or in the conditioned medium of most of the cell lines. Only in MKN1 cells was a low amount of EGF detected in the cellular extract; this EGF level was very close to the detection limit of the assay.

These findings indicate that AREG is expressed in the cetuximab-sensitive cell lines, AZ521 and MKN1, suggesting a positive predictive value of the ligand in gastric cancer cells. By contrast, due to the low detected EGF levels, no correlation could be established between the presence of EGF and the cetuximab responsiveness of the cell lines that were studied.

Effect of cetuximab treatment on AREG levels in AZ521 and MKN1 cells. To examine the role of the EGFR signaling pathway in the regulation of AREG, the levels of secreted AREG were determined after the treatment of cells with cetuximab or combinations of EGF and cetuximab. The AREG concentrations were determined in the conditioned medium of the cetuximab-sensitive cell lines, AZ521 and MKN1, using ELISA.

As shown in Fig. 3, cetuximab treatment caused a significant decrease in the level of secreted AREG in the MKN1 cells,

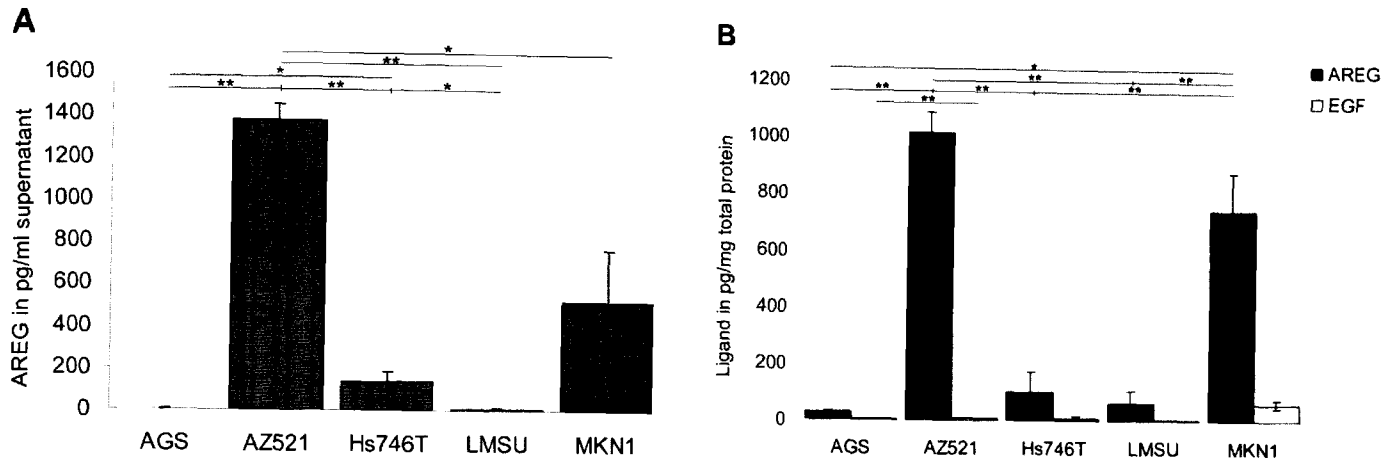

Figure 2. The levels of AREG and EGF in the cellular extracts and conditioned media of the gastric cancer cell lines. Gastric cancer cells were cultivated for 24 h. Subsequently, (A) the concentrations of the EGFR ligands AREG and EGF in the conditioned cell culture media and (B) in the cellular extracts were analyzed by ELISA. The mean value of three independent experiments is shown. The error bars indicate the means ± SD. P-values at significance levels of <0.05 and <0.01 are indicated by (*) and (**), respectively.

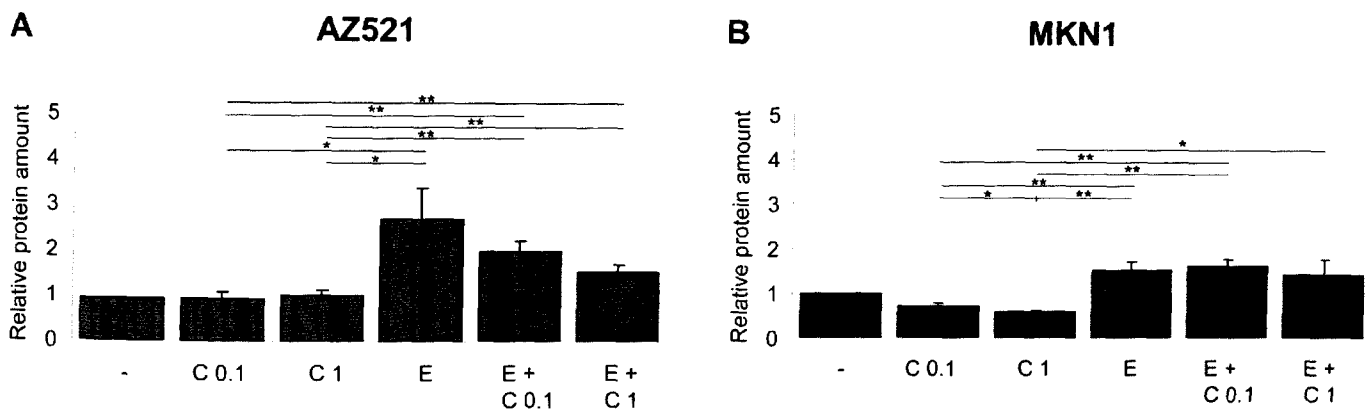

Figure 3. Effect of cetuximab and EGF treatment on the soluble AREG content in the AZ521 and MKN1 cell lines. For evaluation of the soluble AREG level, (A) AZ521 and (B) MKN1 cells were treated for 6 h with cetuximab or EGF alone or in combination. The content of soluble AREG in the conditioned medium was evaluated by ELISA. The values were normalized to those of the untreated control. The mean value of three independent experiments is shown. The error bars indicate the means ± SD. P-values at significance levels of <0.05 and <0.01 are indicated by (*) and (**), respectively. C 0.1, 0.1 μg/ml cetuximab; C 1, 1 μg/ml cetuximab; E, 5 ng/ml EGF; E + C 0.1, 5 ng/ml EGF + 0.1 μg/ml cetuximab; E + C 1, 5 ng/ml EGF + 1 μg/ml cetuximab.

but no such decrease was observed in the AZ521 cells. In both cell lines, the addition of exogenous EGF to the cells resulted in an increase in AREG secretion. This EGF-induced AREG secretion was blocked by simultaneous treatment of the cells with increasing concentrations of cetuximab to some extent in the MKN1 cells and almost completely in the AZ521 cells. By contrast, Hs746T cells did not respond to EGF or cetuximab treatment (data not shown).

These results demonstrate that the EGFR signaling pathway itself is involved in the regulation of the ligand AREG. This important finding suggests that there exists an autoregulatory mechanism in gastric cancer cell lines.

Analysis of EGFR expression, localization and activation. Ligand binding results in EGFR dimerization, stimulation of its tyrosine kinase activity and activation of downstream signaling cascades. The total and activated levels of EGFR were determined in the five gastric cancer cell lines using immunoblot and flow cytometry analyses.

The analysis of the expression level of EGFR in the different cell lines by western blotting revealed the following order: MKN1 > LMSU = Hs746T > AGS = AZ521 (Fig. 4A). Essentially the same order was obtained when the surface localization of EGFR was analyzed with flow cytometry (Fig. 4B).

The activation level of EGFR was determined by western blot analysis of the level of EGFR phosphorylation on tyrosine residue Y1068 (Fig. 4C). The following order of the EGFR activation levels was obtained: Hs746T >> AZ521 = MKN1 > AGS = LMSU. Together, these findings demonstrate that the studied cell lines express EGFR at considerably different levels and that the expression and activation levels of EGFR are not correlated.

Effect of EGF and cetuximab treatment on the phosphorylation of EGFR. The effects of EGF and cetuximab on the expression and activation of EGFR were determined using western blot analysis.

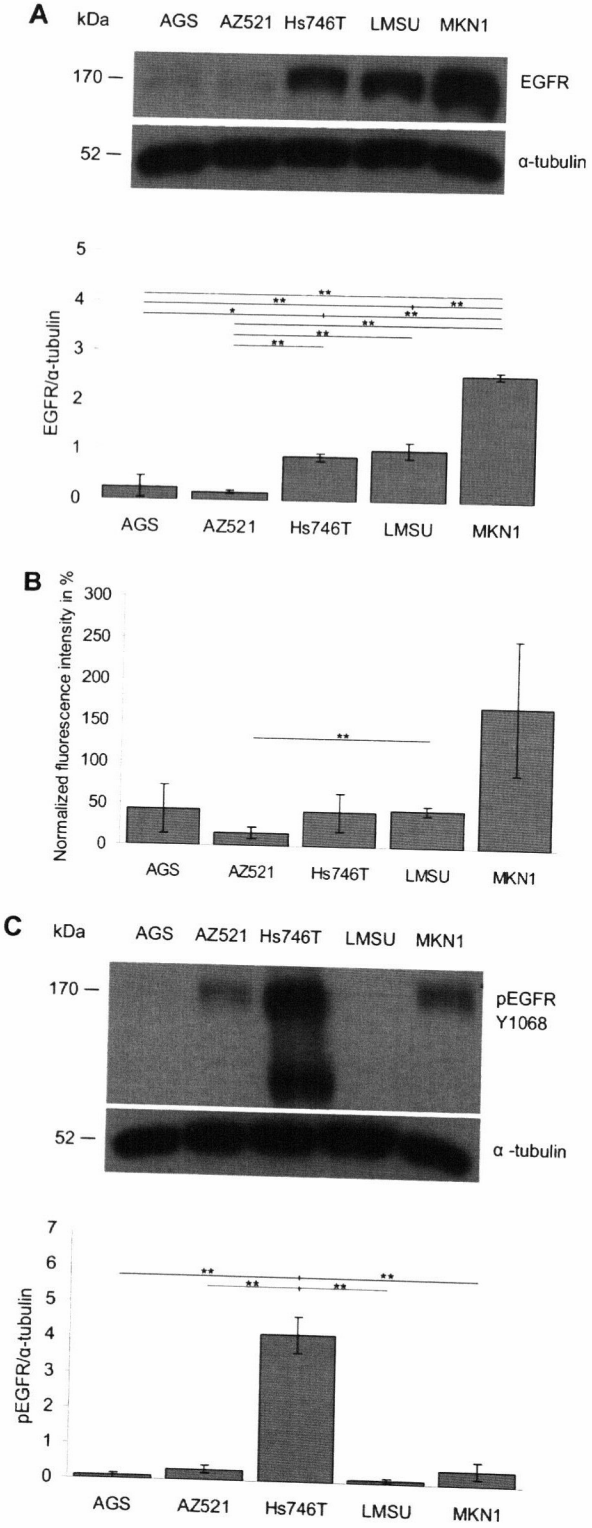

Figure 4. EGFR expression, localization and activation in gastric cancer cell lines. The expression levels of (A) EGFR were determined in the total cell lysates by western blot analysis, using α-tubulin as the loading control. The average expression levels of EGFR were determined by densitometric analysis and calculated in relation to the α-tubulin level. The mean value of three independent experiments is shown. The error bars indicate the means ± SD. (B) Flow cytometry analysis revealed the surface localization of EGFR in all considered cell lines. The normalized fluorescence intensities of 2-3 independent experiments are shown (± SD). Specific fluorescence intensities for EGFR were calculated by normalizing the geometric means of the EGFR-stained cells to the isotype control antibody-stained cells. (C) Phosphorylated EGFR (pEGFR, Y1068) was detected in the total lysates of cells by western blot analysis using α-tubulin as the loading control. The depicted results are representative of three independent experiments. The mean expression levels of pEGFR were quantified using densitometric analysis and were calculated in relation to the levels of α-tubulin (± SD). P-values at significance levels of <0.05 and <0.01 are indicated by (*) and (**), respectively.

Table I. Genetic alterations in hotspot mutation regions of key components of the EGFR signaling pathway in gastric cancer cell lines.

Mutation	AGS	AZ521	Hs746T	LMSU	MKN1
EGFR[a]	WT	WT	WT	WT	WT
KRAS[b]	**G12D**[c]	WT	WT	WT	WT
PIK3CA[d]	WT	WT	WT	WT	**E545K**[e]
BRAF[f]	WT	WT	WT	WT	WT

[a]Exons 18, 19 and 21; [b]exon 2; [c]previously described by Kim *et al* (47); [d]exons 9 and 20; [e]previously described by the Sanger Institute (www.sanger.ac.uk); [f]V600E. WT, wild-type. Bold letters indicate the mutations: *KRAS* **G12D** in AGS cells and *PIK3CA* **E545K** in MKN1 cells.

The detection of EGFR phosphorylated on tyrosine residue Y1068 revealed that EGFR was activated by EGF in the AGS, AZ521, LMSU and MKN1 cell lines and that this EGF-induced activation of EGFR was blocked by cetuximab in a concentration-dependent manner (Fig. 5A, B, D and E). By contrast, Hs746T cells were EGF- and cetuximab-non-responsive (Fig. 5C). In all cell lines, the EGFR expression levels remained essentially unchanged during all treatment conditions.

Together, EGF and/or cetuximab had only minor effects on the degree of EGFR phosphorylation in the Hs746T cell line, whereas EGFR activation was modulated by the treatment in the four other cell lines (AGS, AZ521, LMSU and MKN1).

Analysis of the MET activation. A number of studies have suggested a predictive role for MET in EGFR inhibitory therapy (36-38). To determine the role of MET activation in our gastric cancer model, the phosphorylation status of the MET receptor was determined using western blot analysis. Detection of MET phosphorylated on tyrosine residue Y1234/1235 revealed that the activation of MET was very strong in Hs746T cells, whereas no signals were detected in the AGS, AZ521, LMSU and MKN1 cell lines (Fig. 6).

Mutation analysis. EGFR, KRAS, BRAF and PI3K are key components of the EGFR-signaling pathway, and oncogenic alterations in these genes are related to the response to EGFR-targeting therapeutics. Therefore, hotspot mutation regions in these genes were analyzed in the panel of gastric cancer cell lines.

In detail, for *BRAF*, the cell lines were screened for the activating mutation V600E. Exons 18, 19 and 21 of *EGFR*, exon 2 of *KRAS* and exons 9 and 20 of *PIK3CA* were sequenced.

The presence of the *KRAS* mutation G12D in AGS cells has been reported previously, and we confirmed this finding. We also confirmed the presence of the recently described *PIK3CA* mutation, E545K, in MKN1 cells. No further genetic alterations were identified (Table I).

Model for predicting cetuximab sensitivity in gastric cancer cell lines. In our study, we found that the gastric cancer cell lines, AZ521 and MKN1, were cetuximab-sensitive, whereas the AGS, Hs746T and LMSU cell lines were found to be cetuximab-resistant based on the results of the XTT cell viability assay.

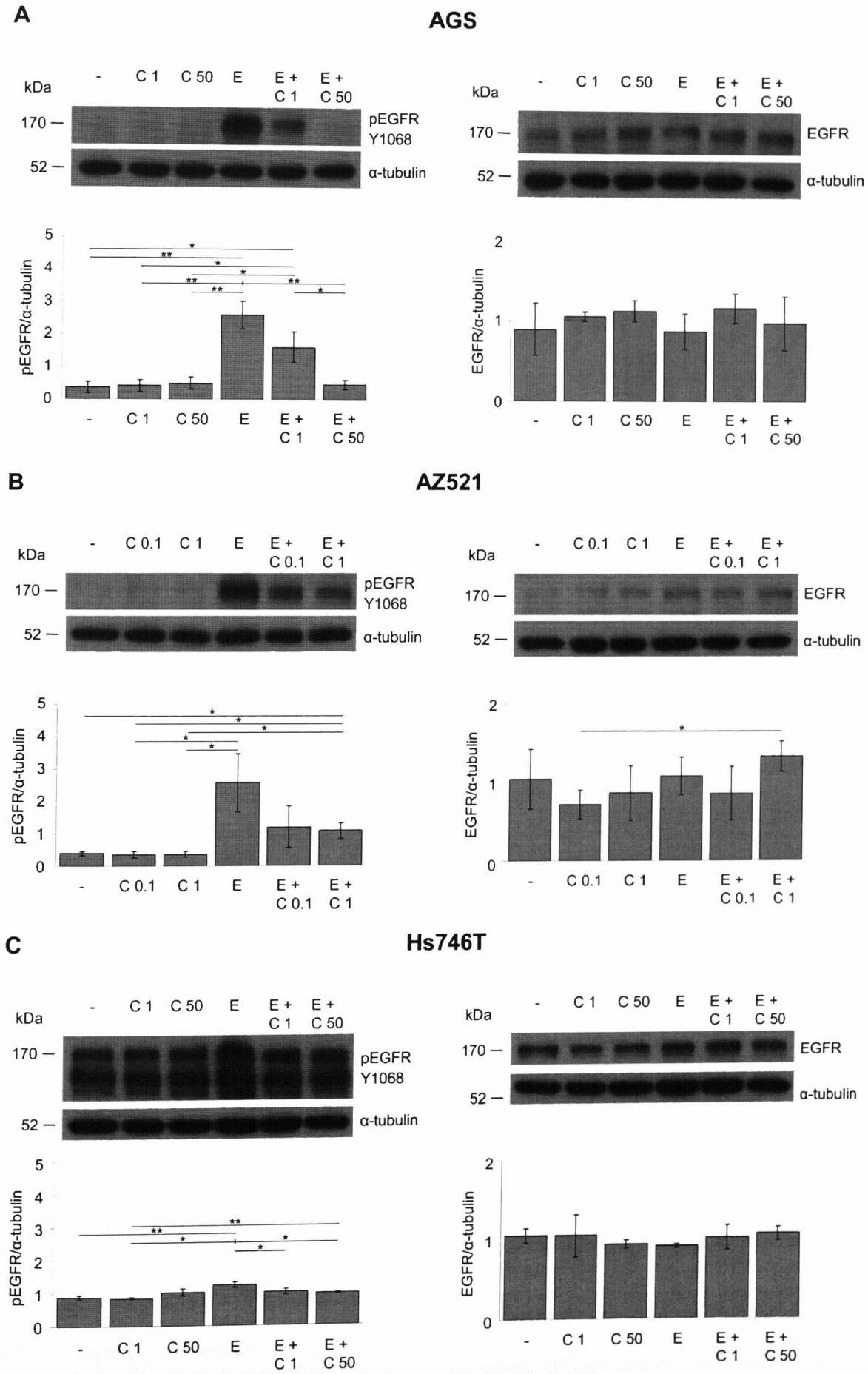

Figure 5. A-C. Effect of cetuximab and EGF treatment on EGFR activation and expression in the gastric cancer cell lines. The levels of activated and total EGFR were determined after treatment of the cells with cetuximab and/or EGF by western blot analysis with a pEGFR-specific antibody (Y1068) and a total EGFR antibody. α-tubulin was used as the loading control. The average phosphorylation levels of EGFR were determined by densitometric analysis and calculated in relation to α-tubulin. The mean value of three independent experiments is shown. P-values at significance levels of <0.05 and <0.01 are indicated by (*) and (**), respectively.

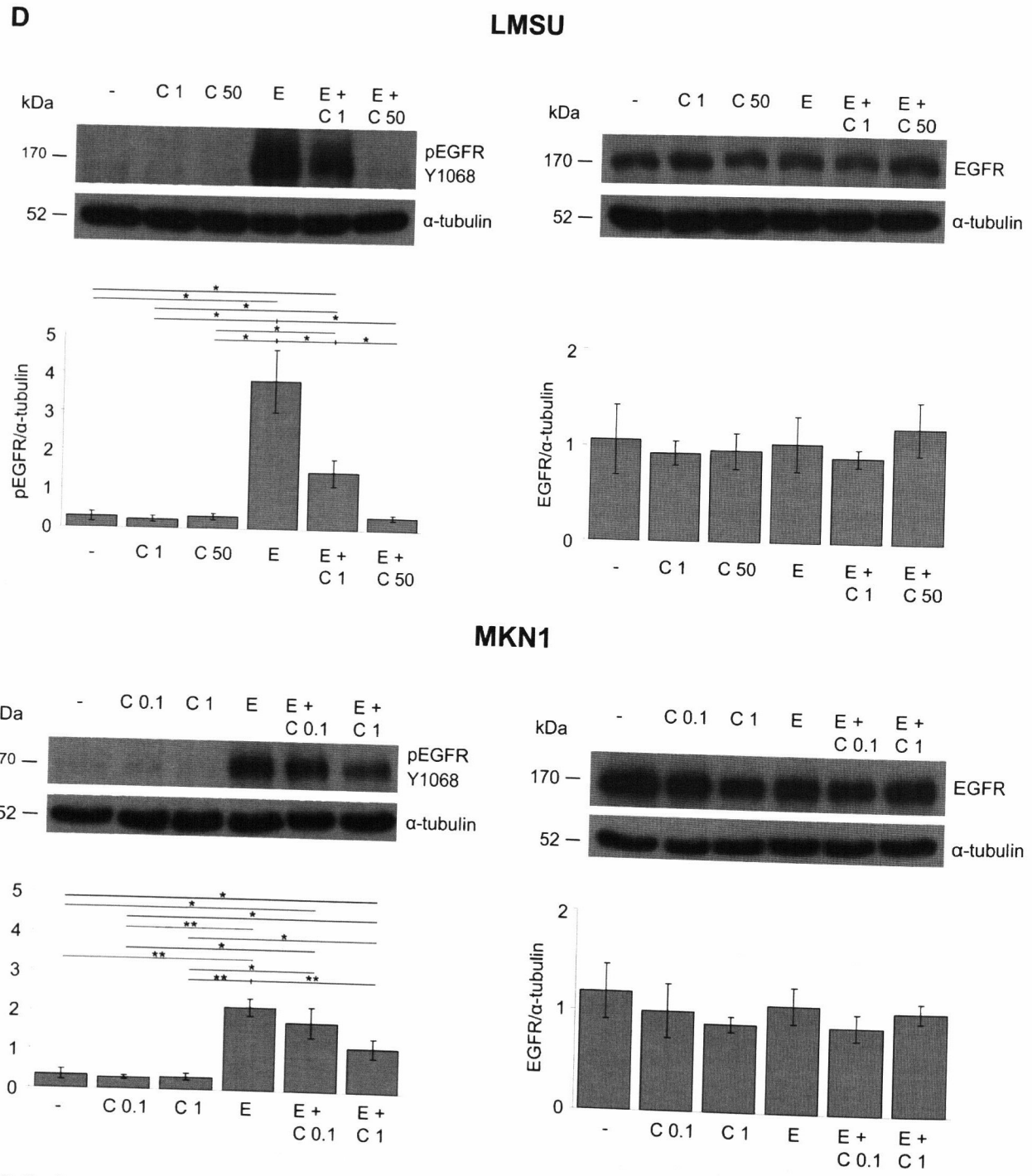

Figure 5. D-E. Continued. C 0.01, 0.01 μg/ml cetuximab; C 1, 1 μg/ml cetuximab; C 50, 50 μg/ml cetuximab; E, 5 ng/ml EGF; E + C 0.1, 5 ng/ml EGF + 0.1 μg/ml cetuximab; E + C 1, 5 ng/ml EGF + 1 μg/ml cetuximab; E + C 50, 5 ng/ml EGF + 50 μg/ml cetuximab. Stimulation time: 3 min.

The cetuximab responsiveness of the AZ521 and MKN1 cells was associated with AREG expression and secretion. The cetuximab resistance of the AGS cells is most likely due to the *KRAS* mutation, whereas the cetuximab insensitivity of Hs746T cells may be explained by MET activation. Together, we were able to explain the cetuximab response of four of the five investigated cell lines.

We propose the model presented in Fig. 7 to predict the cetuximab response of gastric cancer cell lines. To validate this model, we measured the amount of secreted AREG in three additional human gastric cancer cell lines, KATOIII (523 pg/ml in the conditioned medium and 927 pg/mg in the cellular extract), MKN28 (918 pg/ml in the conditioned medium and 106 pg/mg in the cellular extract) and MKN45 (7 pg/ml in the conditioned medium and 168 pg/mg in the cellular extract) (Table II). Among these three cell lines, there was one cetuximab-responsive cell line (MKN28) and two cetuximab-non-responsive cell lines (KATOIII, MKN45) (24). These cell lines have previously been characterized with respect to the *KRAS* mutation and MET activation status (24). MET has been shown to be activated in the cetuximab-resistant cell lines, KATOIII and MKN45, but not in the MKN28 cell line (24). All three cell lines were correctly classified by the model.

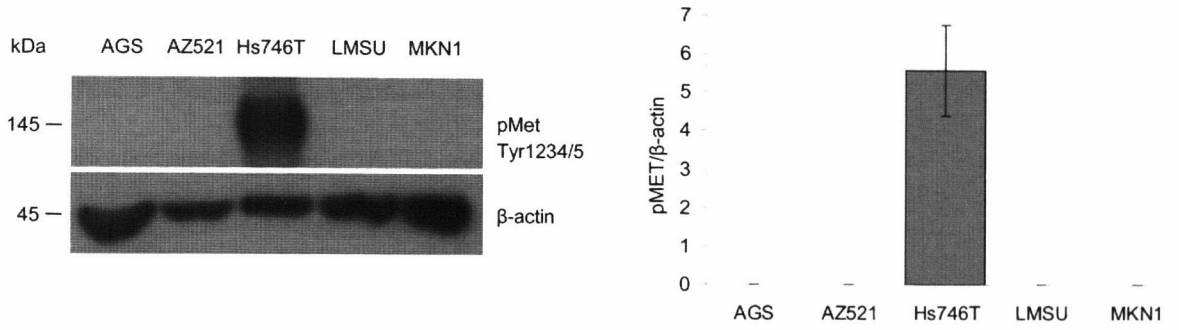

Figure 6. Activation of MET in the gastric cancer cell lines. The concentration of phosphorylated MET (pMET) in the total protein extract of gastric cancer cell lines was evaluated by western blot analysis with a pMET (Y1234/1235) specific antibody. β-actin was used as a loading control. The average phosphorylation levels of MET were determined by densitometric analysis and calculated in relation to the β-actin level. The mean value of three independent experiments is shown. The error bars indicate the means ± SD. P-values at significance levels of <0.05 and <0.01 are indicated by (*) and (**), respectively.

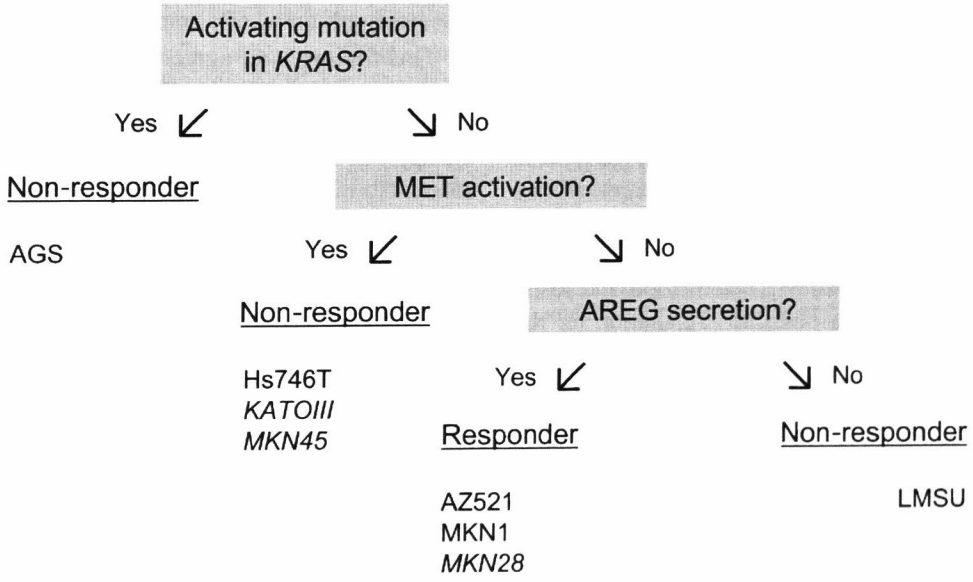

Figure 7. Model for the prediction of the cetuximab responsiveness of the gastric cancer cell lines. Based on three different classification steps, the presence of activating KRAS mutations, MET activation and AREG secretion, five gastric cancer cell lines (AGS, AZ521, Hs746T, LMSU and MKN1) were classified as cetuximab-responsive or cetuximab-non-responsive. To validate this model, three additional gastric cancer cell lines were used (KATOIII, MKN28 and MKN45).

Table II. Molecular characteristics of the gastric cancer cell lines.

Cell line	*KRAS*-status	MET status		AREG secretion	Cetuximab sensitivity[a]
		cMET amplification	MET activation		
AGS	**G12D** (25)	- (39)	-	-	-
AZ521	WT (48)	- (39)	-	+++	+++
Hs746T	WT	++ (39)	+++	+	-
KATOIII	WT (48)	+ (49)	+ (24)	++	- (24)
LMSU	WT	ND	-	-	-
MKN1	WT (48)	- (39,49)	-	++	++
MKN28	WT (48)	- (49)	- (24)	+++	++ (24)
MKN45	WT (48)	++ (39,49)	+++ (24)	-	- (24)

[a]Determined via XTT cell proliferation assay. -, Not detectable; +/++/+++, detectable at low/intermediate/high levels. ND, not done; WT, wild-type. Bold letters indicate the mutation: *KRAS* **G12D** in AGS cells.

Discussion

Responsiveness of gastric cancer cell lines to cetuximab treatment. In this study, the predictive value of several molecular markers for the cetuximab responsiveness of the human gastric cancer cell lines, AGS, AZ521, Hs746T, LMSU and MKN1, was analyzed. The analysis of cell viability after cetuximab treatment identified two cetuximab-responsive cell lines, AZ521 and MKN1, whose metabolic activity was significantly reduced by cetuximab in a dose-dependent manner. The other three cell lines, AGS, Hs746T and LMSU, were not responsive to cetuximab. For the MKN1 and AGS cells, these results are in agreement with the results reported previously by our group (24). All considered cell lines expressed EGFR at different levels.

Model for the prediction of cetuximab responsiveness. In the present study, we performed a detailed molecular analysis of different putative predictive markers for cetuximab responsiveness, including the expression and secretion of the ligands, AREG and EGF, activation of the RTK, MET, and the presence of activating mutations in *KRAS*.

The cetuximab responsiveness of AZ521 and MKN1 cells was associated with AREG expression and secretion. By contrast, the cetuximab resistance of AGS cells was most likely due to the *KRAS* mutation (24). As a number of studies have suggested an association between MET activation and resistance to cetuximab (22-24), one possible explanation for the cetuximab insensitivity of Hs746T cells is the high level of MET tyrosine kinase activity. Notably, the Hs746T cell line harbors a splice site mutation in *cMET*, resulting in the deletion of the juxtamembrane domain (39).

We established a model to facilitate the correct classification of gastric cancer cell lines as cetuximab-responsive and -non-responsive cell lines. In an attempt to establish a hierarchy of predictive molecular markers, the highest priority was allocated to activating *KRAS* mutations. This decision was based on the experience that patients with colorectal cancers lacking oncogenic activation of the EGFR downstream effectors, KRAS, BRAF, PIK3CA and PTEN, are the most likely to benefit from anti-EGFR therapies (21). The second place in the hierarchy was assigned to MET activation. Finally, gastric cancer cell lines lacking activated *KRAS* mutations and MET activation were classified according to their level of secreted AREG. Using this approach, it was possible to correctly classify the cetuximab responsiveness of all five cell lines included in this study (AGS, AZ521, Hs746T, LMSU and MKN1). The reason for the cetuximab resistance of LMSU cells is presently unknown.

The model was validated with three other human gastric cancer cell lines, KATOIII, MKN28 and MKN45, among which one was cetuximab-responsive (MKN28) and two were cetuximab-non-responsive (KATOIII and MKN45) (24). None of these three cell lines harbors a known activating *KRAS* mutation (24). We have previously shown that MET is activated in the cetuximab-resistant cell lines, KATOIII and MKN45, but not in the MKN28 cell line (24). In the present study, we detected high levels of secreted AREG in the MKN28 cells and used the model to classify this cell line as cetuximab-responsive. AREG secretion was not detectable in the cetuximab-resistant cell line MKN45, which falls into the non-responder category due to the elevated MET activation status. Despite the high levels of secreted AREG, the KATOIII cell line was classified as non-responsive due to the high level of activated MET.

In this study, the advantage of the model as a tool to determine the cetuximab sensitivity of gastric cancer cell lines becomes evident. However, it also becomes clear that it is difficult to predict the therapy response by evaluation of a single marker. In order to ensure a correct classification, we suggest defining a panel of predictive markers and establishing a hierarchy among them. Of course, we make no claim of completeness as regards this study.

Positive predictive role of AREG secretion for cetuximab responsiveness. In the present study, we found a positive predictive value of AREG secretion for cetuximab responsiveness when the *KRAS* mutation status and MET activation were taken into account.

This finding is in agreement with the positive predictive role that was attributed to AREG and EREG as markers for the outcome of CRC patients treated with cetuximab and chemotherapy who have no *KRAS* mutations in their tumors (19,20). In contrast to the situation in CRC, in 38 advanced gastric cancer patients treated with cetuximab in combination with oxaliplatin, leucovorin and 5-fluorouracil in a recent clinical phase II trial, no significant correlation between AREG expression and the response rate was found (26). Notably, the level of activated MET was not determined in that study.

One main technical difference between the CRC study and the gastric cancer study is that the AREG mRNA expression level was examined in the CRC tumors, whereas the serum levels of AREG were determined in the gastric cancer patients. When the correlation between tumor mRNA expression and the level of protein in the blood (measured by ELISA) was assessed, only a modest correlation between the systemic protein and tumor mRNA levels of AREG was found (19).

Cetuximab is approved for the treatment of recurrent SCCHN in addition to CRC. For these tumors, the findings regarding the predictive value for AREG are contradictory. In one study, high EGFRvIII and AREG expression levels identified SCCHN patients who were less likely to benefit from combination treatment with cetuximab and docetaxel (40). In a different study that included SCCHN tumors and cell lines, autocrine production of AREG was found to predict sensitivity to both gefitinib and cetuximab in EGFR wild-type cancers (41).

A number of studies have suggested that AREG produced by tumor cells may be involved in the pathogenesis and/or progression of human gastric carcinoma (42,43). Notably, 20 out of 32 tumors (62.5%) expressed AREG mRNA at higher levels than their corresponding normal mucosas. By contrast, no obvious correlation was observed between the AREG mRNA levels and the histological types or tumor staging of gastric carcinoma (43). Additionally, an association between AREG expression and the development of peritoneal carcinomatosis in gastric cancer patients was reported (42).

Recently, it was shown that low levels of the EGFR ligands, EGF and TGFα, in combination with EGFR expression positively correlated with the response rates of gastric cancer patients to a cetuximab/modified FOLFOX6 regimen (26). Considering

the lack of EGF expression in both cetuximab-sensitive and cetuximab-resistant gastric cancer cell lines, we conclude that EGF expression is not of predictive value in our study. This lack of soluble EGF suggests that although EGF is expressed in all cell lines to a certain extent, it is not proteolytically released. Previously, it was proposed that the proteolytic release of EGF is essential for its activity as it cannot act in a juxtacrine manner (44). Due to the absence of soluble EGF, the EGF-based induction of the EGFR signaling pathway most likely plays no role or only a minor role in our gastric cancer cell lines.

Association of MET activation and cetuximab resistance. As mentioned above, a number of studies have suggested a predictive role for MET in EGFR inhibitory therapy (36-38,44,45). As reported previously, the activation of MET was accompanied with the activation of EGFR in the KATOIII and MKN45 cell lines (24). A similar observation was made in the present study: Hs746T cells showed high levels of MET and EGFR activation. Notably, cetuximab had only minor effects on the degree of EGFR phosphorylation in this cell line, whereas EGFR activation was modulated by the treatment in the four other considered cell lines (AGS, AZ521, LMSU and MKN1).

As discussed previously (24), there is a close correlation between MET and EGFR, including physical interaction and ligand-dependent or -independent transactivation, and the co-activation of multiple RTKs in cancer cells results in resistance to single-agent therapy (37,46). Consequently, the co-activation of MET would explain the failure of anti-EGFR therapy in the non-responsive cell lines, Hs746T, KATOIII and MKN45.

In conclusion, in this study, to our knowledge, we present the first model that allows the response of gastric cancer cell lines to cetuximab treatment to be predicted. The considered markers were the status of *KRAS* mutation, MET activation and AREG secretion. Even if cell culture models are too simple to explain the complex *in vivo* situation in patients, we believe that the inclusion of a reasonable number of cell lines and accurate statistical analysis will allow for the generation of hypotheses that can be tested in clinical studies.

Acknowledgements

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