Abstract. Cetuximab has been evaluated as a first-line treatment with conflicting results. The aim of the present study was to investigate the relationship between epidermal growth factor receptor (EGFR) status, and response and survival benefit following cetuximab treatment in gastric cancer (GC). Using 20 patient-derived GC xenograft (PDX) models, the mice (10 mice/model) were randomly assigned into two groups. The control group and treatment group were treated with PBS and cetuximab, respectively. The drug response was evaluated by monitoring tumor growth. Survival benefit was evaluated by comparing the survival curves corresponding to the time for the tumors to reach 600 mm³. Our results revealed that the PDX models treated with cetuximab had better survival than that noted for the non-treated group (P<0.05). The EGFR status was measured by FISH, qPCR, RNAish and immunohistochemistry, respectively. Four cases in the treated group were identified as responsive to cetuximab. EGFR mRNA and protein overexpression were associated with the response to cetuximab (P<0.05). EGFR amplification, mRNA and protein overexpression were associated with prolonged survival in the cetuximab-treated PDX models. Moreover, in the PDX models with EGFR amplification, mRNA or protein overexpression, cetuximab treatment was associated with a better survival compared with that noted in the untreated group in the PDX models (P<0.05), while the survival was not statistically different in the other cases (P>0.05). In conclusion, cetuximab provided survival benefit in the trial. The level of EGFR amplification and overexpression significantly predicted response and survival benefit, particularly the mRNA and protein expression level. A combination of mRNA and protein expression may predict efficacy of cetuximab more efficiently.

Introduction

Gastric cancer (GC) is an aggressive malignancy and remains a major health issue (1). Chemotherapy improves survival and quality of life compared with the best supportive care, but the median overall survival remains poor (2). There is an urgent need for more effective target agents for treating this disease. Cetuximab is a recombinant human/mouse chimeric monoclonal antibody against the epidermal growth factor receptor (EGFR). Cetuximab was found to enhance the effect of oxaliplatin in hypoxic GC cell lines (3). Several phase II trials have evaluated cetuximab as a first-line treatment in combination with various chemotherapy regimens (4-6). However, Erbitux in combination with Xeloda and cisplatin in advanced esophago-gastric cancer (EXPAND; NCT00678535) did not significantly increase progression-free survival (PFS) in patients with advanced GC (7).

In vitro and in vivo antitumor activity of cetuximab in human GC cell lines is related to EGFR expression and mutational phenotype (8,9). EGFR mutations have been proven to be the most effective biomarker for the prediction of superior efficacy for EGFR tyrosine kinase inhibitors (TKIs) (10-12). The potential use of EGFR expression as a marker has been widely investigated, with conflicting results (13). Moreover, gene copy
number gain of EGFR is also a poor prognostic biomarker in GC (8). Unlike HER2 in GC, the predictive value of increased EGFR copy number for tumor response is controversial (14,15). Similarly, the relationship between the level of EGFR amplification and the outcome of EGFR-positive GC treated with first-line chemotherapy with cetuximab remains unclear.

At present, there has been an increase in using experimental models to predict the clinical activity of agents and discover predictive biomarkers. A large collection of patient-derived tumor xenografts (PDXs) reflects the diversity of tumors in patient populations. We established GC PDXs by transplanting surgically removed tumor tissues from patients into immunocompromised BALB/c nude mice via subcutaneous inoculation, to assess drug activity. Moreover, our previous data also suggested that cases with a GC subtype with EGFR amplification and overexpression benefit from cetuximab treatment (16). In the present study, our aim was to determine whether the level of EGFR amplification significantly predicts increased survival and response to therapy in GC treated with cetuximab-based chemotherapy. We investigated the activity of cetuximab in 20 GC-PDX models. After the therapeutic responders and non-responders were identified, the correlation between EGFR amplification, mRNA and protein expression level and tumor response to cetuximab therapy in the GC xenografts were analyzed. Moreover, we also investigated the survival of the GC PDX models in the cetuximab-treated and control groups in regards to the different levels of DNA amplification, mRNA and protein expression.

Materials and methods

Patient and tumor samples. All of the cases with freshly and surgically removed tumor tissues included in the present study were diagnosed and surgically treated at Peking University Cancer Hospital. The tumor-node-metastasis (TNM) stage was classified according to the 7th edition of the classification recommended by the American Joint Committee on Cancer (AJCC) (17). All of the tumors were not previously treated with chemotherapy or EGFR inhibitors. This investigation was performed after approval by the Ethics Committee of Peking University Cancer Hospital.

Antitumor activity evaluation. The subcutaneous engraftment of patient tumor fragments into immunocompromised mice was previously described (18). When the tumor volume reached 100-150 mm³, the mice were randomly grouped into two groups of five mice with a similar average tumor volume. Immediately after grouping, the control group was treated with vehicle [phosphate-buffered saline (PBS), weekly intraperitoneal injection or i.p. for 2 weeks], and the treatment groups were injected with cetuximab (weekly i.p. injection for 2 weeks, 50 mg/kg; Merck KGaA, Darmstadt, Germany). The tumor growth was monitored twice weekly, and ΔT/ΔC value was calculated for assessing tumor response to the treatment (ΔT = tumor volume change in the treatment group and ΔC = tumor volume change in the control group). The total number of mice used for the xenografts was 200 (10 mice/model for 20 PDX models). Survival benefit was evaluated by comparing the survival curves corresponding to the time for the tumor to reach 600 mm³ (19,20). All procedures were carried out under sterile conditions at Crown Bioscience SPF facility and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Crown Bioscience (Crown Bioscience IACUC Committee).

Immunohistochemistry (IHC) staining. Four-micrometer sections from formalin-fixed paraffin-embedded tissues were mounted on poly-L-lysine-coated slides, and then deparaffinized in xylene and rehydrated through alcohol to distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 15 min at room temperature. After pressure cooking the slides in 10 mmol/l EDTA (pH 8.0) for 3 min, the sections were incubated overnight at 4°C with mouse rabbit anti-human EGFR antibody (Cell Signaling Technology, Inc., Boston, MA, USA) at final dilution of 1:200. Primary antibodies were detected using a two-step EnVision System (Dako, Glostrup, Denmark). Positive and negative immunohistochemistry controls were routinely used. For negative controls, the primary antibody was replaced by non-immune mouse serum to confirm its specificity. Moreover, we used an internal positive control in immunohistochemistry for quality assurance. The test specimens were then independently scored by three investigators in a blinded fashion: score 0 is when there was no specific membrane staining within the tumor, and positive when there was any staining of the tumor cell membrane above the background level. The positive cases were further classified into 1+, 2+ and 3+ based on the staining intensity of the membrane.

EGFR gene copy numbers and mRNA expression detection. Quantitative RT-PCR was performed to determine the relative EGFR gene expression level for all of the samples. Extracted mRNA was subjected to amplification using human EGFR-specific primers by TaqMan q-PCR (assay ID, Hs01076078_m1; Applied Biosystems, Foster City, CA, USA). The human GAPDH gene was used as a reference (assay ID, Hs99999905_m1; Applied Biosystems). Each sample was run thrice. Expression of each gene was represented as the ratio of expression of each target gene mRNA to that of GAPDH mRNA. Moreover, Affymetrix HG-U219 array was also performed following a standard protocol (http://media.affymetrix.com/support/downloads/manuals/3_iva_ 
express_kit_manual.pdf) to detect EGFR mRNA expression.

In addition, EGFR gene copy numbers were determined by quantitative PCR. Briefly, the same genomic DNAs were subjected to amplification by TaqMan qPCR. The primers for EGFR (assay ID, Hs04960197_cn) and RNase P as endogenous reference (part no. 4401631) were purchased from Applied Biosystems. The raw data were transferred to CopyCaller software and analyzed.

FISH. Quantitative assessment of EGFR copy number was also investigated by FISH. Dual-color, dual-target FISH assays were carried out using the EGFR Spectrum Orange/CEP7 Spectrum Green Probe (Vysis, Des Plaines, IL, USA). Three-micrometer thick tissue sections were treated with the procedure provided by fluorescence in-situ hybridization (FISH) detection kit (DakoCytomation, Glostrup, Denmark). Briefly, samples were
placed in pretreatment solution for 30 min at 96°C, and digested with pepsin solution for 30 min at room temperature. Tissue sections, covered with 10-µl probe solution, were incubated at 75°C for 5 min to co-denature the EGFR and chromosome seven α-centromeric (CEP7) probes and allowed to hybridize overnight at 37°C. Co-denaturation and hybridization were carried out sequentially. Post-hybridization stringency wash was carried out in a water bath at 65°C for 10 min. Then, tissue sections were covered with DAPI II (Vysis) for chromatin counterstaining. EGFR was visualized as a red signal with a standard tetramethyl Rhodamine isothiocyanate (TRITC) filter, CEP7 as a green signal with a fluorescein isothiocyanate (FITC) filter, and nuclei as a blue signal with a DAPI filter. Representative images of the samples were acquired and then analyzed.

Two independent observers scored at least 100 non-overlapping interphase nuclei for the number of copies of EGFR and CEP7 by use of predefined scoring guidelines. EGFR status was scored as the presence of 2.5 or more signals/nucleus, i.e., EGFR copy number ≥2.5.

### RNA in situ hybridization assays in FFPE samples.

**In situ hybridization (ISH)** was performed using QuantiGene® ViewRNA ISH Tissue Assay (Affymetrix, Santa Clara, CA, USA) following the manufacturer's protocol. Briefly, FFPE slides were prepared according to the procedure described. Then, sections were rehydrated and incubated with Proteinase K. Standard probe design software was used to design specific oligonucleotide probe sets for detecting target genes. A no-probe sample was utilized as a negative control per the Affymetrix manual's recommendations. The signal was amplified before incubation with labeled probes and visualized. Hybridized target mRNAs were visualized using confocal fluorescent microscopy, or by bright field microscopy.

The test specimens were then independently scored by three investigators in a blinded fashion: score 0 is when there was no specific staining within the tumor, and positive when there was any staining of the tumor cell membrane above the background level. The positive cases were further classified into 1+, 2+ and 3+ based on the staining intensity.

### Statistical analysis.

In order to investigate correlations between tumor response (ΔT/ΔC value) and CN/mRNA/protein expression, the ΔT/ΔC value, DNA and mRNA expression value was coded as one for expression levels ranked as at or below the 25th percentile of the total gene expression, two for levels above the 25th and at or below the 50th percentiles, three for levels above the 50th and at or below the 75th percentiles, and four for levels above the 75th percentile. Spearman rank order correlations were performed to analyze the correlation between tumor response and CN/mRNA/protein expression. Hazard ratios from univariate Cox regression analysis were used to determine whether the factor was associated with death. Protective genes were defined as those associated with a hazard ratio for death of <1; risk genes were defined as those associated with a hazard ratio for death of >1. Kaplan-Meier analysis was used to compare survival of the two groups of PDX models (by 600 mm²) with the log-rank test. In all analyses, P<0.05 was considered to indicate statistical significance, and all tests were two-tailed.

### Correlation between the tumor response and EGFR status in the cetuximab-treated GC xenografts.

The tested GC-PDXs fell into two distinct categories according to the drug activity: 4 of 20 (20%) responded with a nearly complete response (ΔT/ΔC value <0) to cetuximab treatment; 16 of 20 (80%) did not, 1 of 20 had a partial response and 15 had complete resistance (ΔT/ΔC value >30%).

The correlations between tumor response and the level of DNA amplification, mRNA and protein expression in GC xenografts treated with cetuximab were analyzed. Most of the IHC 3+ cases and mRNA overexpression by RNAish and qPCR had a significant positive correlation with the tumor response to cetuximab (Spearman's, RNAish, P=0.001; EGFR/GAPDH, P=0.001; EGFR IHC score, P=0.003), while EGFR CN, either detected by qPCR or FISH (EGFR/CEP7), was not significantly correlated with the EGFR CN and tumor response (EGFR CN detected by qPCR, P=0.398; FISH, P=0.119, 0.232, respectively; Table I).

### Table I. Correlation between the DNA, RNA and protein expression level of EGFR and cetuximab response.

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Correlation</th>
<th>P-value</th>
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<tbody>
<tr>
<td>DNA CN</td>
<td>0.20</td>
<td>0.398</td>
</tr>
<tr>
<td>FISH (EGFR/CEP7)</td>
<td>0.28</td>
<td>0.232</td>
</tr>
<tr>
<td>FISH (EGFR)</td>
<td>0.36</td>
<td>0.119</td>
</tr>
<tr>
<td>mRNA expression</td>
<td>0.671</td>
<td>0.001</td>
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<td>RNAish</td>
<td>0.707</td>
<td>0.001</td>
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<tr>
<td>U219 intensity</td>
<td>0.720</td>
<td>&lt;0.001</td>
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<tr>
<td>qPCR (EGFR/GAPDH)</td>
<td>0.630</td>
<td>0.003</td>
</tr>
<tr>
<td>Protein</td>
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<td></td>
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<tr>
<td>EGFR IHC score</td>
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EGFR, epidermal growth factor receptor; IHC, immunohistochemistry.

### Results

**EGFR status in the GC xenografts.** We established GC-PDX models by surgically transplanting removed tumor tissues from GC patients into immunocompromised BALB/c nude mice subcutaneously. qPCR, FISH (EGFR and EGFR/CEP7), RNAish and immunohistochemical staining were performed to investigate EGFR expression in the 20 GC xenografts. Amplification of EGFR by FISH was determined by EGFR or the EGFR/CEP7 ratio with a range 1.9 to 15 or 0.83 to 2 (Fig. 1A). The ranges of EGFR CN by qPCR were 1.4-104.9. The ranges of mRNA by qPCR (EGFR/GAPDH) were 0.00-13.00. RNAish assay showed that 5 of 20 GC xenografts were EGFR positive; one of them showed strong positive (Fig. 1B). For the 20 cases included in the analysis, 7 were IHC 0, 7 were 1+, 2 (10%) were 2+, and 4 (20%) were 3+ (Fig. 1C).

The correlations between tumor response and the level of DNA amplification, mRNA and protein expression in GC xenografts treated with cetuximab were analyzed. Most of the IHC 3+ cases and mRNA overexpression by RNAish and qPCR had a significant positive correlation with the tumor response to cetuximab (Spearman's, RNAish, P=0.001; EGFR/GAPDH, P=0.001; EGFR IHC score, P=0.003), while EGFR CN, either detected by qPCR or FISH (EGFR/CEP7), was not significantly correlated with the EGFR CN and tumor response (EGFR CN detected by qPCR, P=0.398; FISH, P=0.119, 0.232, respectively; Table I).
Survival of the GC PDX models. Survival of the GC PDX were compared between the cetuximab-treated and control group. Our results suggested that the PDX models treated with cetuximab had longer survival compared with that noted in the control cases (median, 19.5 days vs. not reached). This difference was statistically significant (log-rank, P=0.011; Fig. 2A). In the univariate Cox analyses, cetuximab was significantly associated with survival [hazard ratio 0.371; 95% confidence interval (CI), 0.164 to 0.838; P=0.017]. Moreover, the PDX models responsive to cetuximab had longer survival than those in the non-responsive cases (log-rank, P=0.049; Fig. 2B).

Survival and different level of DNA amplification, mRNA and protein expression in cetuximab-treated cases. For the 20 cases, 6 were EGFR CN ≥4 as detected by qPCR, 14 were EGFR/CEP7 ≥1 as detected by FISH, and 7 were EGFR ≥2.5 by FISH. PDX models treated with cetuximab considered CN <4 had shorter survival than those patients with CN ≥4 (median, 22.0 days vs. not reached; P=0.050; Fig. 3A). This difference was statistically significant by qPCR, while there was no statistically significant difference detected by FISH (EGFR/FISH, median, 24.0 days vs. not reached, log-rank; P=0.221; Fig. 3B).
Then, we evaluated EGFR mRNA and protein expression level and the survival following treatment with cetuximab. Of these, 5 cases had EGFR mRNA overexpression as detected by RNAish or qPCR (EGFR/GAPDH). Their survival was longer than that of those with a lower mean (RNAish, median, 24.0 days vs. not reached, log-rank; P=0.139; qPCR, log-rank; P=0.019, respectively; Fig. 3C and D). The EGFR protein expression of 4 patients was IHC 3+; survival of these cases was better than that of those with a lower expression (log-rank; P=0.049; Fig. 3E).

**Survival and cetuximab therapy in different EGFR copy number subgroup.** Amplification: for both EGFR/CEP7 ratio value, one was used as the optimal cut-off value that discriminated between the GC PDX model with longer survival and those who more probably may respond to cetuximab-based therapy. As illustrated in Fig. 4A in the cases with EGFR ≥2.5, the median survival of the cetuximab-treated group was significantly longer than that of the non-treated group (log-rank; P=0.046). Similarly, cases with EGFR CN >4 by qPCR showed an increased and statistically significant benefit in survival (median, 21.0 vs. not reached; log-rank, P=0.013; Fig. 4C). However, either in the group with EGFR <2.5 by FISH or EGFR CN <4 by qPCR, the survival rate was not statistically significant between the cetuximab-treated and control group (EGFR/FISH, median, 52.0 days vs. not
reached, log-rank; \( P=0.089 \); EGFR CN/Q-PCR median, 18.0 vs. 22.0 days, log-rank; \( P=0.105 \), respectively; Fig. 4B and D).

**Survival and cetuximab therapy in the different EGFR mRNA expression level subgroups.** The mRNA expression of EGFR was detected by RNAish and EGFR/GAPDH. In the EGFR expression-positive cases (detected by RNAish), the median survival of the cetuximab-treated group was significantly longer than that of the non-treated group (median, 19.5 days vs. not reached; log-rank, \( P=0.003 \); Fig. 4E), while in the RNAish-negative cases, the survival rate did not differ significantly (median survival, 20.0 vs. 24.0 days; \( P=0.205 \); Fig. 4F).

Similarly, in the EGFR/GAPDH \( \geq 0.2 \) group, those in the cetuximab-treated group showed a longer survival rate than those of the control cases (median survival; \( P=0.015 \); Fig. 4G), while in the EGFR/GAPDH <0.2 group, the survival rate did not significantly differ in the cetuximab-treated and control cases (median survival, 18.0 vs. 22 days; \( P=0.139 \); Fig. 4H).

**Survival and cetuximab therapy in the different EGFR protein expression level subgroup.** Moreover, the survival rate and cetuximab therapy in the GC PDX models with different levels of EGFR protein expression was also investigated. A score of 2+ was used as the optimal EGFR IHC score cut-off value. In the EGFR protein overexpression cases, the median survival rate of the cetuximab-treated group was significantly longer than that of the non-treated group (median, 19.5 days vs. not reached; log-rank, \( P=0.025 \); Fig. 4I), while in the group with EGFR expression IHC score <2 cases, the survival rate did not differ significantly (median, 20.0 vs. 24.0 days; log-rank, \( P=0.155 \); Fig. 4J).

**Combined detection of DNA amplification, mRNA and protein overexpression and the survival rate of tumor response to cetuximab.** DNA amplification, mRNA and protein expression are commonly detected by FISH, RNAish and IHC in clinical testing. Combined detection is more meaningful for drug use. We found that 4 of the patients were positive for combined detection, which was consistent with the nearly complete response cases; the others were negative. In the combined positive cases, the median survival rate of the cetuximab-treated group was significantly longer than the negative cases (log-rank, \( P=0.049 \); data not shown).

**Discussion**

The potential use of EGFR expression as a marker has been widely investigated, with conflicting results. In the present study, our aim was to determine whether the level of EGFR gene amplification, mRNA and protein level could significantly predict some benefit in the survival and response to cetuximab in GC xenografts. EGFR DNA amplification was detected by EGFR/CEP7 ratio and qPCR, mRNA expression was detected by qPCR, RNAish, EGFRRIU219 and protein overexpression was detected by IHC. EGFR protein and mRNA expression levels allowed us to identify and discriminate those patients with prolonged survival. We showed, for the first time to the best of our knowledge, how the level of EGFR gene amplification, mRNA and protein expression may be used as a predictive factor for response to cetuximab-based treatment and also for survival benefit in this subset of GC patients through PDX models.

Benefit from the addition of an anti-EGFR agent to chemotherapy could not be confirmed in the phase III trials comparing chemotherapy with and without anti-EGFR agent, including the randomized EXPAND and REAL3 trials. An important point is that neither of these trials selected patients based upon biomarkers (7,21). Various authors have suggested that both IHC and FISH should be used to determine the HER2 target chemotherapy status in GC (22). However, EGFR status evaluation method mostly focused on the gene mutations of downstream genes, such as KRAS and BRAF. EGFR protein expression was found to be associated with the survival rate in GC (23). In the present study, we tested the RAS mutation status. There was no RAS mutation in these tumor samples. Our results showed that cases with high EGFR mRNA expression and immunohistochemistry score were more prone to response to cetuximab. EGFR mRNA and protein overexpression were associated with the survival rate in the cetuximab-treated PDX models. Moreover, in the PDX models derived from mRNA or protein overexpression cases, the survival rate of the cetuximab-treated PDX models was significantly longer than that noted in the control group, while the survival was not statistically different in the other cases. The potential use of EGFR expression as a marker has been widely investigated in other cancers, such as NSCLC, with conflicting results. Previous report suggested that EGFR overexpression is associated with improved response, longer time to progression and improved survival in NSCLC patients treated with gefitinib (24). Biomarker analysis of the BR.21 study showed that survival among patients with high EGFR expression was longer in the erlotinib arm vs. the placebo arm, whereas a limited advantage of erlotinib treatment was noted in patients with EGFR IHC-negative tumors (25). This result is similar with ours detected in GC (25).

Gene copy number gain of EGFR is a poor prognostic biomarker in GC (8). At present, there are few studies that have focused on the association of EGFR gene copy number and efficacy of cetuximab chemotherapy, including evaluation of EGFR gene copy number as a predictive biomarker for the efficacy of cetuximab in combination with chemotherapy in the first-line treatment of recurrent and/or metastatic squamous cell carcinoma of the head and neck: EXTREME study (15). The association of EGFR and tumor response to cetuximab should be further studied. In the present study, similar to Her2 amplification (2), we also found that EGFR gene copy number was a predictive biomarker for the efficacy of cetuximab in a GC PDX model, similar with the results in previous studies (26). Compared to EGFR DNA amplification, mRNA and protein overexpression may be more accurate to determine the tumor response to cetuximab. This may be due to other regulatory methods of EGFR expression, such as epigenetics.

In summary, the level of EGFR gene amplification significantly predicted the sensitivity to therapy and the survival rate in GC cases treated with cetuximab-based chemotherapy. This result supports the proposed combined use of IHC and in situ hybridization in this setting. The study of the level of DNA amplification, mRNA and protein expression as a continuous biomarker is arguably a more rational approach for selecting patients more likely to benefit from anti-EGFR-based therapies.
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References


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