Antitumour Activity of S-1 in Combination with Cetuximab on Human Gastric Cancer Cell Lines *In Vivo*

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Abstract. *This study aimed to assess the antitumour effect of a combination of cetuximab (Erbitux, a chimeric anti-epidermal growth factor receptor (EGFR) monoclonal antibody) and S-1, an oral 5-fluorouracil prodrug, on gastric cancer cell lines in vivo. Gastric cancer cell lines (SC-2 and SC-4) were transplanted subcutaneously into nude mice. In both cell lines, which have high EGFR expression and harbour K-ras wild-type alleles, treatment with a combination of cetuximab and oral S-1 resulted in significantly higher antitumour activity than treatment with cetuximab or S-1 alone. To investigate this potentiation of antitumour activity, the expression levels of thymidylate synthase (TYMS) were measured following administration of cetuximab. Cetuximab induced a decrease in expression of TYMS mRNA and protein. These findings suggest that cetuximab-mediated down-regulation of TYMS enhances the antitumour effect of S-1 and provide a rationale for designing novel combination chemotherapy regimens for patients with advanced gastric cancer.*

The human epidermal growth factor receptor (EGFR) is a well-established drug target in cancer of the colon, and head and neck, as well as in non-small cell lung cancer (NSCLC). EGFR is a member of the transmembrane tyrosine kinase receptor family that triggers the Ras/Raf/mitogen-activated protein kinase pathway and controls cell growth, differentiation, and proliferation (1, 2). The association between poor prognosis and high EGFR expression in head and neck squamous cell carcinoma has been known for years (3). Expression of EGFR has been reported as a marker for poor prognosis in various malignancies such as prostate, colorectal, breast, and NSCLC (4-7). S-1, an orally administered 5-fluorouracil (5-FU) prodrug, is approved for the treatment of gastric, colorectal, breast, head and neck, NSCLC, pancreatic, and biliary cancer in Japan and gastric cancer in Europe. Recently, a large biomarker analysis of the 'Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer' (ACTS-GC) study, a randomized phase III trial, which demonstrated that adjuvant treatment with S-1 after D2 dissection for locally advanced gastric cancer was more effective than surgery alone in East Asian patients (8), also showed that EGFR expression level was associated with worse outcomes in gastric cancer patients (9). However, it is not yet clear whether anti-EGFR drugs, such as cetuximab, afford these patients a clinical benefit.

To provide a rationale for further combination chemotherapy regimens for EGFR-positive gastric cancer with EGFR-targeting drugs and S-1, we conducted *in vivo* experiments and basic studies on the mechanisms of action of the combination therapy.

Materials and Methods

Chemicals. Cetuximab was purchased from Bristol-Myers Squibb Co. Ltd. (Princeton, NJ, USA). Tegafur, gimeracil, and potassium oteracil were obtained from Taiho Pharmaceutical Co. Ltd (Tokyo, Japan). S-1 is a combined formulation of 1 molar tegafur, 0.4 molar gimeracil, and 1 molar potassium oteracil. Anti-TYMS monoclonal antibody for immunoblot analysis of proteins was prepared by Taiho Pharmaceutical Co. Ltd (10), and antibodies against EGFR were purchased from Dako Japan Inc. (Tokyo, Japan).

Tumour xenografts. Human colonic tumour SC-2 and SC-4 xenografts were provided by the Central Institute for Experimental Animals (Kawasaki, Japan) and maintained by serial subcutaneous implantation into the right axillae of Balb/c$^{nu/nu}$ nude mice (Charles River Laboratories Japan Inc., Japan) at 3-week intervals.

Immunostaining. Formalin-fixed, paraffin-embedded tumour tissues were sliced into 4-μm sections. The tissue specimens on the slide were then deparaffinized, and endogenous peroxidase was inactivated. After 2-h exposure to 0.53 μg/ml of anti-EGFR monoclonal antibody at room temperature, they were allowed to react with a reagent containing horseradish peroxidase-labelled dextran-bound anti-rabbit IgG goat polyclonal antibody (Dako Japan Inc., Tokyo, Japan). The chromogenic substrate used for detection was 3,3'-diaminobenzidine (DAB). Slides were counterstained with hematoxylin.

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DNA extraction and determination of K-ras status. Genomic DNA was extracted from frozen samples using the AllPrep DNA/RNA Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions, as described previously (11). The *K-ras* mutation status was determined using the Peptide nucleic acids (PNA) -clamp real-time PCR TaqMan assay, as previously described (12).

Antitumour experiments. Fragments of SC-2 and SC-4 tumours, approximately 3-mm in size, were implanted subcutaneously into the right axillary region of the mice. When the estimated tumour volume ($0.5 \times \text{length} \times \text{width}^2$) reached 100-200 mm^3, the tumour-bearing mice were allocated randomly to a test group (day 0, n=7). S-1 (at 10, 8.3, or 6.4 mg/kg) was administered orally once a day from day 1 to day 14, and cetuximab (20 mg/kg) was injected intraperitoneally on days 1, 4, 8, and 11. The tumour volume [$0.5 \times$ (major axis) $\times$ $(\text{minor axis})^2$] was measured twice a week throughout the treatment period (15 days), and relative tumour volume (RTV) was calculated as follows: RTV=(mean tumour volume during therapy)/(mean tumour volume at the beginning of therapy). The antitumour effects of S-1, cetuximab, and the combination were estimated by the following equation: mean inhibition rate of tumour growth (IR, %)=[1-(mean RTV of drug-treated group/mean RTV of control group) $\times$100]. A parallel experiment was performed with cetuximab to obtain tumour samples for gene expression analysis and Western blotting. All animal experiments were carried out in accordance with the Guidelines for the Welfare of Animals in Experimental Neoplasia.

RNA isolation and RT-PCR. Total RNA was extracted from frozen samples using the AllPrep DNA/RNA Mini Kit (Qiagen Inc.) according to the manufacturer's instructions. Gene expression levels were determined using TaqMan real-time PCR (Applied Biosystems, Foster City, CA, USA) as described previously (13). First-strand cDNA was synthesized from total RNA using the high capacity cDNA Reverse transcription kit (Applied Biosystems) in 20-μl reaction volumes for TaqMan real-time PCR analysis, according to the manufacturer's instructions. cDNA (10 ng/μl) was added to 9.15 μl RNase-free water, 12.5 μl 2× TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25 μl 20× Primer Probe mix. β-actin (*ACTB*) was used as the endogenous control gene (Applied Biosystems). Assay IDs for *TYMS* and *ACTB* were Hs00426591_m1 and Hs99999903_m1, respectively. PCR amplification was carried out using the Prism 7900HT Sequence Detection System (Applied Biosystems) under the following thermal cycler conditions: 2 min at 50˚C and 10 min at 95˚C for 40 cycles (15 s at 95˚C and 1 min at 60˚C). Relative TYMS gene expression was calculated by comparing delta Ct values.

Western blot analysis for TYMS protein expression. TYMS protein expression was evaluated by Western blot analysis. Total protein from fresh-frozen tumour tissue was extracted using a protein extraction reagent (T-PER; Pierce Biotechnology, Rockford, IL, USA) supplemented with protease inhibitors (Halt Protease Inhibitor Cocktail kit; TAKARA BIO Inc. Tokyo, Japan). Tissue lysates (50 μg protein/sample) were loaded into 4-12% sodium dodecyl sulphate polyacrylamide gradient gels. After electrophoresis, the separated proteins were electrotransblotted onto polyvinylidene difluoride membranes (Immobilon-P membrane, Millipore, Billerica, MA, USA). After blocking, membranes were probed with anti-human TYMS monoclonal mouse antibody. The proteins were detected using horseradish peroxidase–conjugated antibodies (Pierce) followed by enhanced chemiluminescence. The intensity of luminescence was quantified using an image analysis system (LAS-3000; Fuji Film, Tokyo, Japan).

Statistical analysis. The statistical significance of differences between groups with and without treatment was assessed using Dunnett's test and the Intersection Union-test (IUT) procedure.

Results

Immunohistochemistry for EGFR. As shown in Figure 1, gastric cancer cell lines SC-2 and SC-4 were evaluated for EGFR protein expression by immunohistochemistry. EGFR was predominantly detected in the membrane and the cytoplasm.

K-ras status. Delta Ct values after PNA-clamp PCR were 8.25 and 8.26 for SC-2 and SC-4, respectively, whereas the delta Ct value for the positive control gDNA harbouring *K-ras* mutated alleles was –1.8. This means that PNA was able to securely hybridise to the wild-type DNA (wtDNA) template and inhibit the amplification of wtDNA at the *K-ras* allele of SC-2 and SC-4. In the case of the mutant type gDNA, however, PNA showed no inhibitory effect. Thus, the *K-ras* status of both cell lines was found to be wild-type.

In vivo antitumour activity. Nude mice were used to confirm the effectiveness of S-1 plus cetuximab on gastric tumours *in vivo*. S-1 and cetuximab tended to suppress tumour growth in all treated groups when compared with control (Figure 2, Table I). In both SC-2- and SC-4-xenograft-bearing mice, the maximum reduction in body weight after administration of either of the drugs or the combination was below 18%; thus, S-1, cetuximab, and the combination were found to be well tolerated. S-1 alone (10 mg/kg/day) had significant antitumour activity against SC-2, with an IR of 24.6%. Treatment with cetuximab alone resulted in an IR of 20.2%. However, the addition of cetuximab to S-1 resulted in antitumour activity that was significantly higher than that of both S-1 and of cetuximab alone ($p<0.05$), with an IR of 36.4%. Similar results were seen for the SC-4 cell line, where S-1 alone (10 mg/kg/day) led to a significant IR in SC-4 tumours (38.7%) and cetuximab alone gave an IR of 20.7%. Again, the addition of cetuximab to S-1 resulted in a significantly higher antitumour activity than treatment with either S-1 or cetuximab alone ($p<0.05$), with an IR of 47.6%.

TS expression level after cetuximab treatment. The *in vivo* antitumour activity prompted us to further explore the basic mechanisms underlying the effects of the combination. We observed that cetuximab treatment induced a decrease in *TYMS* mRNA expression in the xenografts of the two gastric cancer cell lines (Figure 3A). Treatment with 20 mg

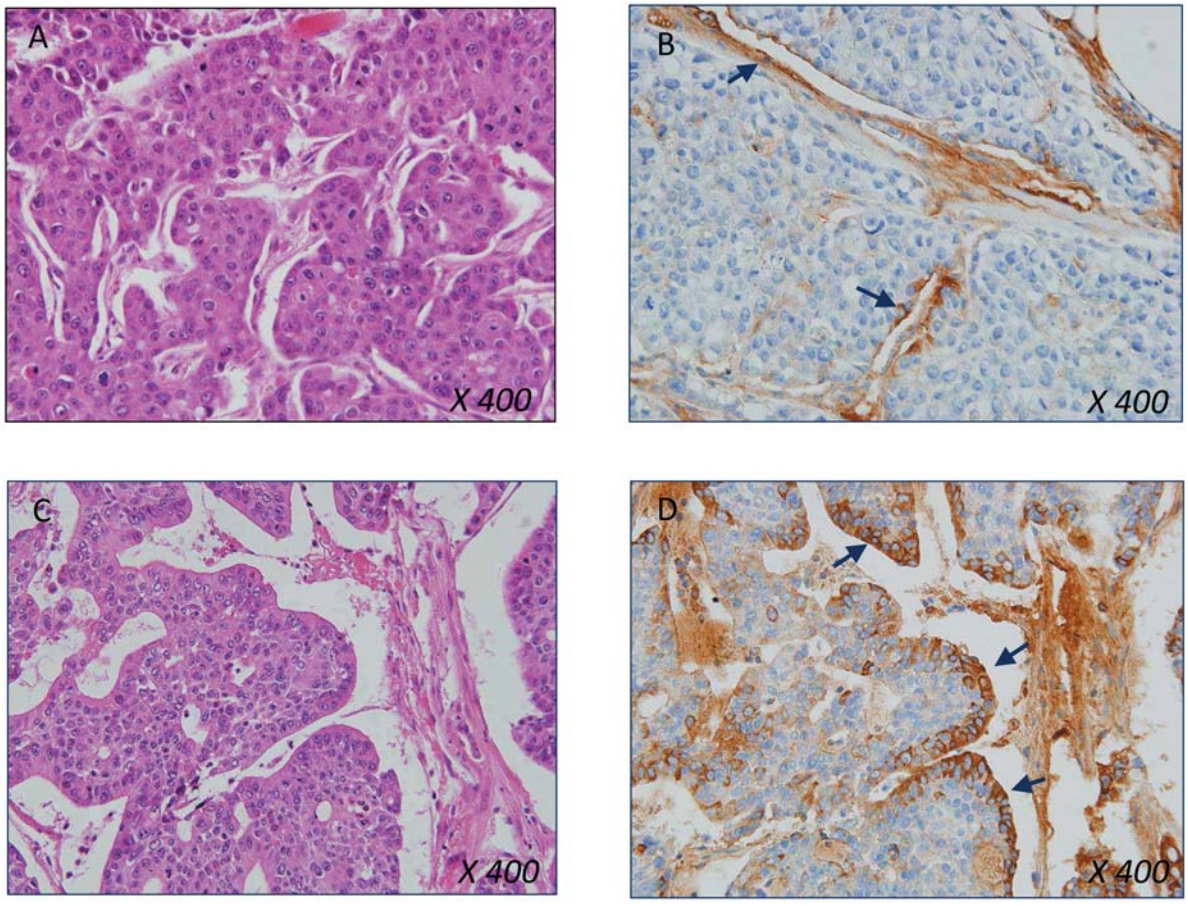

Figure 1. *Epidermal growth factor receptor immunohistochemistry in SC-2 and SC-4 gastric cancer xenografts. A and C: Hematoxylin-eosin-stained SC-2 and SC-4 cells. Immunohistochemical staining shows high expression of EGFR in SC-2 (B) and SC-4 (D) xenografts. Arrows indicate EGFR-positive cells.*

cetuximab on day 0 reduced the *TYMS* mRNA expression level in SC-2 and SC-4 by 55% and 40%, respectively, on day 1. On day 2, the *TYMS* mRNA level in SC-2 xenografts recovered to the initial level; however, the *TYMS* mRNA level in SC-4 remained 20% lower. Real-time PCR data were confirmed by Western blot (Figure 3B). TYMS protein levels were decreased on day 1 after cetuximab treatment in both cell lines. In contrast to mRNA, the protein level was still reduced on day 2 in xenografts from both cell lines.

Discussion

In this study, we were able to show that combining cetuximab and S-1 against EGFR-positive human gastric cancer cells *in vivo* is beneficial.

S-1, an orally administered fluoropyrimidine formulation is widely used in clinical practice in Japan as standard therapy, especially for gastric cancer. Cetuximab, a chimeric monoclonal antibody directed against EGFR, is indicated in patients with EGFR-expressing *K-ras* wild-type metastatic colorectal cancer and in patients with squamous cell carcinoma of the head and neck; however, its indications do not yet extend to gastric cancer patients. This is the first study to report that the combination of S1 and cetuximab resulted in a significantly enhanced activity against gastric cancer cells *in vivo*.

Previous studies on various human tumour models revealed that inhibition of the EGFR signalling pathway can enhance the antitumour activity of cytotoxic drugs such as topoisomerase I and II inhibitors (14-16) and platinum agents (17) and irradiation (18). Skvortsov et al: also reported that cetuximab inhibits TYMS in colorectal cells; however, no correlation was seen between the level of cetuximab-induced TYMS down-regulation and response to 5-FU, neither alone nor in combination with cetuximab (19). This merits further consideration. We demonstrated that cetuximab treatment of two gastric cancer cell lines significantly reduced TYMS expression levels. TYMS is a key enzyme in DNA synthesis, catalysing the conversion of deoxyuridylate to

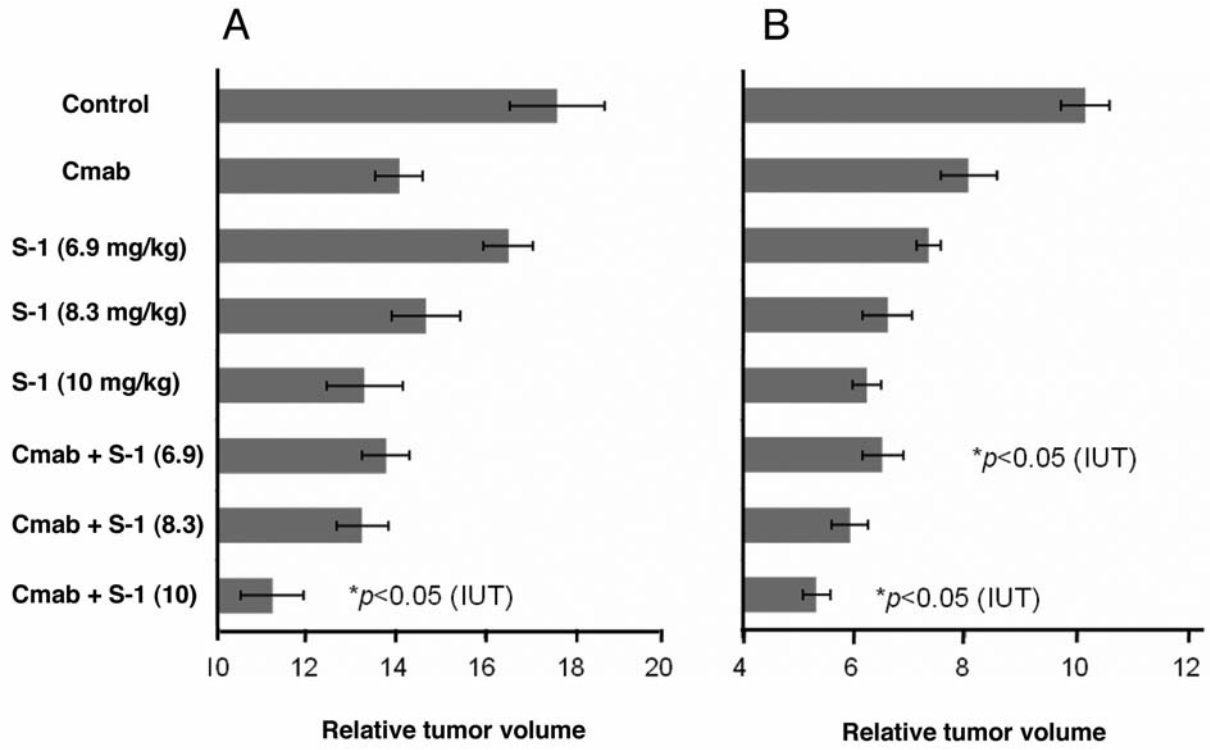

Figure 2. *Antitumour effect of S-1 combined with cetuximab(Cmab) on SC-2 (A) and SC-4 (B) human gastric cancer xenografts. S-1 (6.9, 8.3, and 10 mg/kg/day) was administered orally for 14 consecutive days, and cetuximab (20 mg/kg) was administered intraperitoneally on days 1, 4, 8, and 11. *p<0.05, Significantly different from S-1 alone and cetuximab alone (IUT statistical procedure).*

Table I. *Antitumour effect and body weight changes in mice after treatment with cetuximab, S-1, and their combination for SC-2 and SC-4 human gastric cancer xenografts.*

Xenograft	Drug	Dose (mg/kg/day)	IR (%)	Body weight change† (%, mean and SD)
SC-2	Control	–	–	–9.34±3.2
	Cetuximab	20	20.2	–3.51±4.4
	S-1	6.9	6.2	–7.57±4.6
	S-1	8.3	16.8	–10.1±5.2
	S-1	10	24.6	–9.78±7.6
	S-1+Cetuximab	6.9+20	21.9	–5.63±3.9
	S-1+Cetuximab	8.3+20	24.9	–8.42±4.3
	S-1+Cetuximab	10+20	36.4 *	–7.54±1.8
SC-4	Control	–	–	–8.9±3.9
	Cetuximab	20	20.7	–9.4±3.6
	S-1	6.9	27.7	–13.4±4.4
	S-1	8.3	35.0	–14.6±3.5
	S-1	10	38.7	–13.7±6.4
	S-1+Cetuximab	6.9+20	35.8*	–15.5±3.6
	S-1+Cetuximab	8.3+20	41.6	–17.8±5.6
	S-1+Cetuximab	10+20	47.6*	–11.5±4.8

IR: Tumour growth inhibition rate on day 15 on the basis of relative tumour volume(RTV) was calculated according to the following formula: IR(%) = [1–(mean RTV of the treated group)/(mean RTV of the control group)] ×100. †: Body weight (BW) change on day 15 was calculated according to the following formula: BWC (%)=[(BW on day 15) - (BW on day 0)]/(BW on day 0) ×100. *Overall maximal $p<0.05$ by Intersection-Union Test procedure.

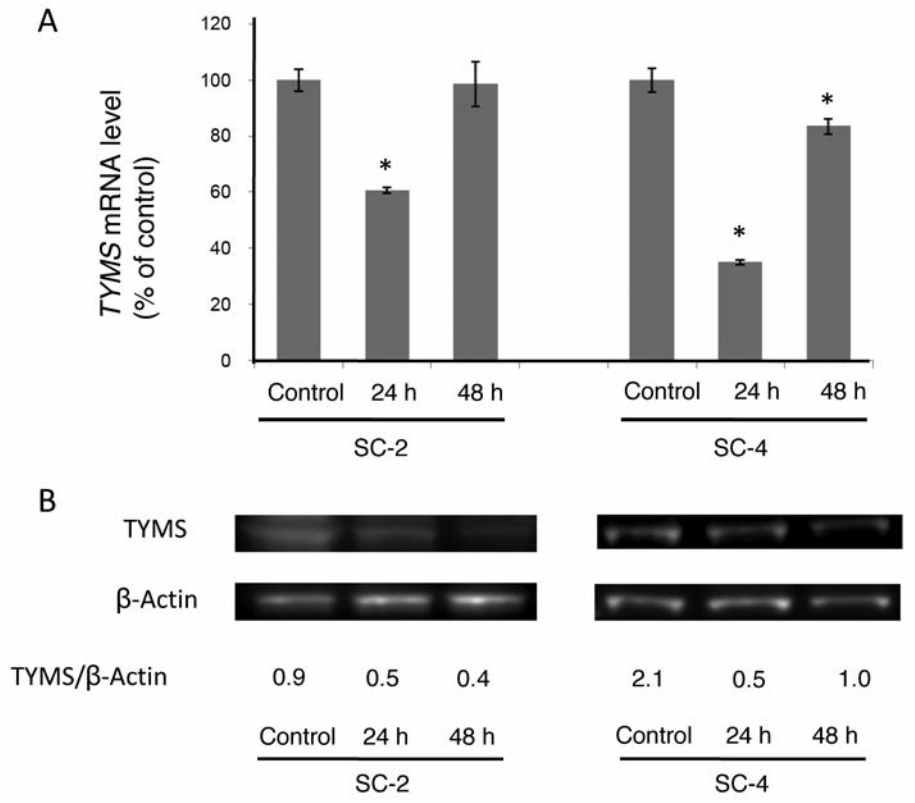

Figure 3. *Effect of cetuximab administration on thymidilate synthase (TYMS) mRNA expression in SC-2 and SC-4 human gastric cancer xenografts. Twenty-four and 48 hours after cetuximab (20 mg/kg) administration, tumours were removed and homogenized to measure mRNA expression level and protein level. A: TYMS mRNA level compared with control cells. Columns, average mRNA using 7 tumours per treatment group; bars, SE. *p<0.05 (versus control by t-test). B: Representative immunoblots from SC-2 and SC-4 xenografts probed for TYMS and β-actin.*

deoxythymidylate. Over the past few decades, a considerable number of studies have been directed at the 5-FU metabolic pathway, including TYMS, regarding the efficacy of fluoropyrimidine treatment for gastric cancer. A low level of TYMS expression in human solid tumours is thought to predict a better response to 5-FU (20-23). Moreover, TYMS is reported to be a factor predicting poor prognosis in gastric cancer. These reports suggest that down-regulating TYMS may help overcome 5-FU resistance in a clinical setting. It is therefore reasonable to suppose that the down-regulation of TYMS expression we have demonstrated here in gastric cancer *in vivo* experiments could be a key mechanism enhancing the antitumour effect of 5-FU after cetuximab administration. A recent large biomarker analysis of the ACTS-GC study showed that EGFR expression was associated with worse outcomes in gastric cancer patients. This reveals the necessity for effective chemotherapy regimens in patients with such EGFR-overexpressing tumours, with poor outcomes after S-1 monotherapy. Based on the results from our basic experiments, we are now able to see a rationale for further development of chemotherapy regimens for EGFR-positive gastric cancer that combine an EGFR-targeting drug and S-1. The combination of cetuximab and S-1 could be a promising therapeutic strategy for gastric cancer patients with poor prognosis.

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