

Cetuximab-induced insulin-like growth factor receptor I activation mediates cetuximab resistance in gastric cancer cells

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Received December 6, 2013; Accepted September 12, 2014

DOI: 10.3892/mmr.2015.3245

Abstract. Epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor-I (IGF-IR) are frequently overexpressed in gastric cancer cells. However, these cells are resistant to the anti-EGFR monoclonal antibody cetuximab. The aim of the present study was to determine whether cetuximab resistance in gastric cancer cells resulted from activation of the IGF-IR signaling pathway by cetuximab. The results demonstrated that EGFR phosphorylation was markedly inhibited in gastric cancer cell lines (SGC7901 and MGC803) which possessed functional K-ras and BRAF following treatment with cetuximab. However, cetuximab treatment did not diminish cell viability; by contrast, IGF-IR activation was observed. Knockdown of IGF-IR or the use of an IGF-IR inhibitor were found to increase the sensitivity of gastric cancer cells to cetuximab. Furthermore, cetuximab induced phosphorylation of the non-receptor tyrosine kinase c-steroid receptor co-activator (Src). Treatment of gastric cancer cells with a Src inhibitor was shown to significantly reduce cetuximab-induced phosphorylation of IGF-IR as well as Src, which resulted in enhanced sensitivity to cetuximab treatment. In conclusion, the results of the present study demonstrated that cetuximab-induced IGF-IR activation was involved in cetuximab resistance in gastric cancer cells and that Src was an important mediator for IGF-IR activation.

Introduction

Gastric cancer is highly prevalent in East Asia, with 42% of cases occurring in China (1). The median life expectancy of gastric

cancer patients following diagnosis is <1 year; however, combination chemotherapy treatments have the potential to extend the survival rate of advanced stage patients (2-8). Therefore, using chemotherapy in conjunction with effective targeting of key factors may be beneficial for improving the clinical outcome of gastric cancer patients. A phase III clinical trial demonstrated that the use of chemotherapy in conjunction with a human epidermal growth factor receptor 2 (HER2)-specific monoclonal antibody (trastuzumab) significantly improved the overall survival rate of patients with HER2-neu overexpressing gastroesophageal junction cancer compared with that of chemotherapy alone (7). However, overexpression of Her-2 is present in only 10-20% of gastric cancer patients (9) and therefore, this combination treatment may not be beneficial for the majority of patients. Cetuximab (C225), an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, has been widely used in combination with chemotherapy for the treatment of various cancer types, including metastatic colorectal cancers that retain wild-type *K-ras* and *BRAF* genes, squamous cell carcinoma of the head and neck as well as non-small cell lung cancer (NSCLC) (10-12). The majority of gastric cancers overexpress EGFR (13), while retaining wild-type *K-ras* and *BRAF* genes (14,15). Phase II clinical studies have shown that cetuximab in combination with chemotherapy delayed the progression of gastric cancer in patients, with an acceptable response rate (13,16-18). However, two additional trials failed to demonstrate significant improvement in overall patient survival with use of the anti-EGFR antibodies cetuximab or panitumumab in combination with chemotherapy in advanced gastric cancer patients compared with that of chemotherapy alone (19,20). The results of these studies therefore suggested that alternate mechanisms of resistance to anti-EGFR antibodies existed in gastric cancer patients.

Numerous key molecules are involved in the EGFR signal transduction pathway, which is also able to cross-talk with other signaling pathways. In addition to K-ras and BRAF, other molecules influence EGFR signaling pathways, including C-Met and the insulin-like growth factor receptor-I (IGF-IR) signaling pathway (21-23). IGF-IR is a receptor tyrosine kinase, which is overexpressed in numerous types of tumor, such as gastrointestinal carcinomas (24-26). IGF-IR becomes autophosphorylated following the binding of ligands, which

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Key words: insulin-like growth factor receptor-I, cetuximab, steroid receptor co-activator, gastric cancer

stimulates its tyrosine kinase activity and subsequently activates downstream signaling pathways (27). These pathways include the Ras/Raf/mitogen-activated protein kinase and phosphoinositide 3-kinase/Akt pathways, which are the primary downstream mediators of EGFR signaling (28). This therefore suggested that IGF-IR may modulate the sensitivity of gastric cancer cells to anti-EGFR antibodies.

Resistance to cetuximab was reported to be associated with overactivation of baseline IGF-IR in human nasopharyngeal carcinoma cells; in addition, the inhibition of baseline IGF-IR activation increased sensitivity to cetuximab in cutaneous squamous cell carcinoma (29). However, the involvement of cetuximab in the activation of the IGF-IR pathway and inhibition of the EGFR pathway as well as the role of IGF-IR signaling in cetuximab resistance in gastric cancer cells has remained to be elucidated.

The non-receptor tyrosine kinase c-steroid receptor co-activator (Src) was reported to have a crucial role in IGF-IR signaling. Numerous studies have indicated that Src may be an upstream signaling molecule of IGF-IR and EGFR in kidney cells and epididymal cells (30,31). By contrast, certain studies have shown that IGF-IR acts upstream of Src in human prostate cancer DU145 and breast cancer cells (32,33). Src has also been implicated in chemotherapy resistance in gastric cancer (34). However, the involvement of Src in the regulation of IGF-IR signal transduction and thereby cetuximab sensitivity in gastric cancer cells has remained to be elucidated.

The aim of the present study was to investigate the role of cetuximab in the induction of IGF-IR and Src activation in gastric cancer cells in order to determine the mechanisms underlying cetuximab resistance.

Materials and methods

Reagents and antibodies. Cetuximab was obtained from Merck KGaA (Darmstadt, Germany). Src inhibitor 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*] pyrimidine (PP2) was obtained from Sigma (St. Louis, MO, USA). IGF-IR inhibitor OSI-906 was purchased from SelleckBio (Houston, TX, USA). The following antibodies: Anti-EGFR polyclonal antibody, anti-phospho-EGFR (Tyr1068) polyclonal antibody, anti-phospho-Akt (Ser473) polyclonal antibody, anti-IGF-IR monoclonal antibody, anti-phospho-Src (Y416) polyclonal antibody and anti-phospho-IGF-IR (Tyr1131) polyclonal antibody, were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The following antibodies: Anti- β -actin polyclonal antibody, anti-Akt monoclonal antibody, anti-extracellular signal-regulated kinase (ERK)1/2 polyclonal antibody, anti-c-Src monoclonal antibody and anti-phospho-ERK1/2 (Tyr202/ Tyr204) polyclonal antibody, were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell cultures. Gastric cancer SGC7901 and MGC803 cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Mutations were not located in exons 19 or 21 of the EGFR gene in the two gastric cancer cell lines. The cells were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL), 100 U/ml

penicillin and 100 μ g/ml streptomycin (Life Technologies, Inc., Carlsbad, CA, USA) at 37°C under an atmosphere of 95% air and 5% CO₂. Cells were routinely subcultured every two to three days and all cells used for experimental procedures were in the logarithmic growth phase.

Small interfering RNA (siRNA) transfections. IGF-IR siRNAs were obtained from Shanghai Gemma pharmaceutical technology Co., Ltd (Shanghai, China). IGF-IR siRNA was synthesized using the primer 5'-GCATGGTAGCCGAAGATTT-3'. Lipofectamine® 2000 was diluted dropwise into RPMI 1640 and incubated at room temperature for 5 min. IGF-IR siRNA was then added to the diluted Lipofectamine® 2000 and incubated for 20 min. Following 48 h of transient transfection, cells were analyzed using western blot analysis.

Cell viability assay. Cell viability was measured using an MTT assay. Cells were seeded at 3×10^4 /well in 96-well plates and incubated overnight. Cells were then exposed to increasing doses of cetuximab (0.01, 0.1, 1.0 and 10 μ g/ml) for 24 h; following which, 25 μ l MTT solution (5 mg/ml) was added to each well and the cells were incubated for 4 h at 37°C. The cell culture medium was then removed and the cells were lysed in 200 μ l dimethylsulphoxide. Optical density was measured at 570 nm using a microplate reader (Bio-Rad 550; Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis. Cells were washed twice with ice-cold phosphate-buffered saline and solubilized in 1% Triton lysis buffer [1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA); 50 mM Tris-Cl, pH 7.4; 150 mM NaCl; 10 mM EDTA; 100 mM NaF (all purchased from Sinopharm Chemical Reagent, Shanghai, China); 1 mM Na₃VO₄; 1 mM phenylmethylsulfonyl fluoride; and 2 μ g/ml aprotinin (all purchased from Sigma-Aldrich)] on ice and then quantified using the Lowry method (35). Cell lysate proteins were separated using SDS-PAGE and electrophoretically transferred onto a nitro-cellulose membrane (Immoblin-P; Millipore, Bedford, MA, USA). Membranes were blocked using 5% skimmed milk in trimethyl benzene sulfonyl tetrazole buffer (10 mM Tris-Cl, pH 7.4; 150 mM NaCl; 0.1% Tween 20; all purchased from Sinopharm Chemical Reagent) at room temperature for 2 h and incubated with anti-EGFR, anti-IGF-IR, anti-c-Src, anti-ERK1/2, anti-Akt, anti- β -Actin, anti-phospho-EGFR (Tyr1068), anti-phospho-IGF-IR (Tyr1131), anti-phospho-Src (Y416), anti-phospho-ERK 1/2 (Tyr202/ Tyr204) or anti-phospho-Akt (Ser473) primary antibodies at 4°C overnight. The secondary anti-rabbit or mouse monoclonal antibodies (Santa Cruz Biotechnology, Inc.) antibodies (dilution, 1:800) were then added for 30 min at room temperature. Proteins were detected using an enhanced chemiluminescence reagent (SuperSignal Western Pico Chemiluminescent Substrate; Pierce Biotechnology, Rockford, IL, USA) and visualized using the Electrophoresis Gel Imaging Analysis System (DNR Bio-Imaging Systems, Ltd., Jerusalem, Israel).

Colony-forming assay. In brief, 300 cells per well were seeded onto 12-well plates. Following adherence to the plates, cells were exposed to 10 μ g/ml cetuximab, PP2 and OSI-906. On day 14, clones were air dried without RPMI-1640, then stained for 10 min with Giemsa stain (Sigma-Aldrich). The clones

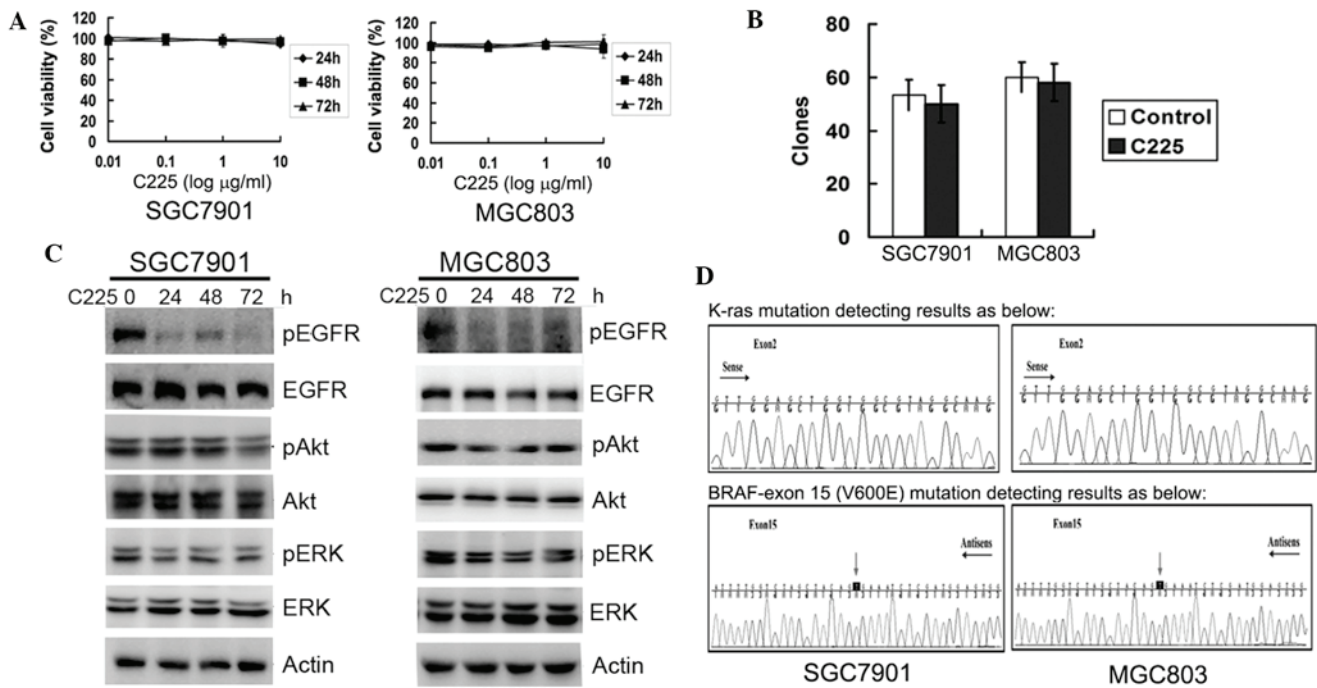


Figure 1. Effects of cetuximab on cell viability, colony formation and EGFR signaling pathways in two gastric cancer cell lines. (A) SGC7901 and MGC803 cells were treated with cetuximab (0.01, 0.1, 1 and 10 $\mu\text{g/ml}$) for 24, 48 and 72 h. Cell viability was measured using an MTT assay. (B) The two cell lines were exposed to 10 $\mu\text{g/ml}$ cetuximab for 14 days and the colony formation was analyzed. (C) Western blot analysis of the EGFR signaling pathways of SGC7901 and MGC803 cells treated with 10 $\mu\text{g/ml}$ cetuximab for 2, 6 and 24 h. (D) Primer sequences for mutation analysis of K-ras and BRAF. EGFR, epidermal growth factor receptor; ERK, extracellular signal-related kinase; p, phosphorylated; C225, cetuximab.

were then washed with running water and air dried again. Clones in each well were counted and images were captured using inverted microscopy (M021; Olympus, Tokyo, Japan).

Statistical analysis. All experiments were performed in triplicate. Values are expressed as the mean \pm standard deviation. Statistical comparisons were made by Student's t-test. SPSS 16.0 software was used for statistical analysis (International Business Machines, Armonk, NY, USA) and $P < 0.05$ was considered to indicate a statistically significant difference between values.

Results

Gastric cancer cell lines are resistant to cetuximab. In order to evaluate the sensitivity of gastric cancer cell lines to cetuximab, SGC7901 and MGC803 cells were treated with increasing concentrations of cetuximab (0.01, 0.1, 1 and 10 $\mu\text{g/ml}$) for 24, 48 and 72 h. Following treatment with cetuximab, the two cell lines exhibited minimal growth inhibition ($<10\%$), which therefore indicated that the cells were cetuximab-resistant (Fig. 1A) and the maximal dose of 10 $\mu\text{g/ml}$ cetuximab was therefore used for the subsequent experiments. As shown in Fig. 1B, the colony forming ability of gastric cancer cells was not affected by cetuximab treatment. Furthermore, in order to determine whether cetuximab had a role in blocking EGFR tyrosine kinase activation, the effect of cetuximab treatment on EGFR, ERK and Akt phosphorylation was examined. Cells were incubated with 10 $\mu\text{g/ml}$ cetuximab for 2, 6 and 24 h. The results demonstrated a marked decrease in EGFR and ERK phosphorylation; however, Akt phosphorylation remained unchanged (Fig. 1C).

In addition, following a mutation analysis of *K-ras* (codons 12 and 13) and *BRAF* (exon 15, V600E) genes, no point mutations were observed in the two cell lines. This therefore indicated that cetuximab resistance was not associated with the mutation of these genes (Fig. 1D). Overall, these results suggested that an alternative pathway mediated cetuximab resistance via activation of Akt in gastric cancer cells.

Cetuximab induces activation of IGF-IR and Src in gastric-cancer cells. SGC7901 and MGC803 cells were exposed to 10 $\mu\text{g/ml}$ cetuximab for 0.5, 2, 6, 16 and 24 h. Western blot analysis revealed that IGF-IR phosphorylation was notably increased in the two cell lines, with peak activation detected at 6 h. Increased Src phosphorylation was also observed in MGC803 and SGC7901 cells, with peak activation detected at 6 and 16 h, respectively (Fig. 2). This suggested that cetuximab may have induced the activation of IGF-IR and Src in gastric cancer cells.

Inhibition of IGF-IR activation or expression increases sensitivity of gastric cancer cells to cetuximab and reduces Src phosphorylation. In order to determine whether IGF-IR signaling induced cetuximab resistance, SGC7901 and MGC803 cells were treated with 10 $\mu\text{g/ml}$ cetuximab in combination with the tyrosine kinase dual insulin receptor and IGF-IR inhibitor OSI-906 (10 μM) for 2 and 6 h. OSI-906 inhibited the cetuximab-induced phosphorylation of Src, IGF-IR and Akt in the two cell lines; however, ERK activation was not altered (Fig. 3A). Cetuximab treatment in combination with OSI-906 decreased cell viability in SGC7901 and MGC803 cells, respectively, by ~ 25 and 27% compared to treatment with cetuximab alone (Fig. 3B). Furthermore, a colony-forming assay of

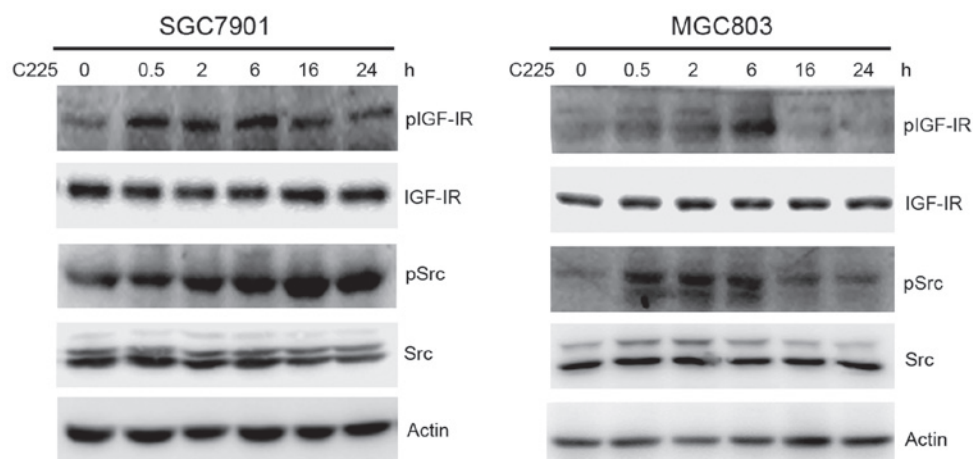


Figure 2. Cetuximab activates IGF-IR and Src. Cells were treated with 10 μ g/ml cetuximab for 0.5, 2, 6, 16 and 24 h. Western blot analysis was then used to detect protein expression levels of IGF-IR and Src. β -actin was used as an internal control. IGF-IR, insulin-like growth factor receptor 1; Src, steroid receptor co-activator; p, phosphorylated; C225, cetuximab.

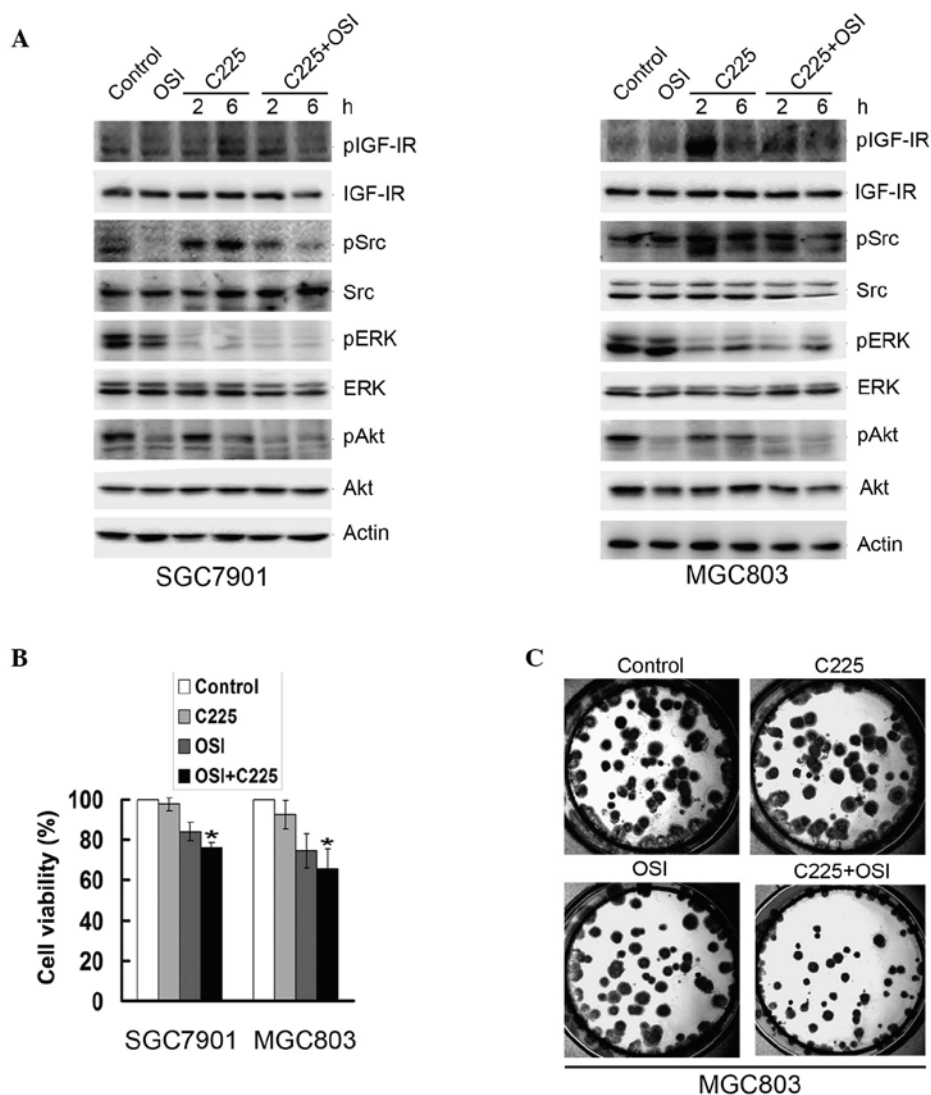


Figure 3. OSI-906 suppresses IGF-IR and Src phosphorylation and enhances the growth inhibitory effects of cetuximab in gastric cancer cells. (A) SGC7901 and MGC803 cells were pretreated with or without the IGF-IR inhibitor OSI-906 (10 μ M) for 1 h and then incubated with 10 μ g/ml cetuximab for 2 and 6 h. Western blot analysis was then used to detect protein expression levels of phosphorylated and non-phosphorylated IGF-IR, Src, ERK and Akt. (B) SGC7901 and MGC803 cells were incubated with 10 μ g/ml cetuximab with or without 10 μ M OSI-906 for 48 h. Cell viability was then assessed using an MTT assay. * P <0.05 treatment with combination cetuximab and OSI-906 vs. cetuximab alone. (C) Cells were treated with 10 μ g/ml cetuximab and 10 μ M OSI-906 alone or in combination for 14 days and a colony forming assay was performed. IGF-IR, insulin-like growth factor receptor 1; Src, steroid receptor co-activator; ERK, extracellular signal-related kinase; p, phosphorylated; C225, cetuximab; OSI, OSI-906.

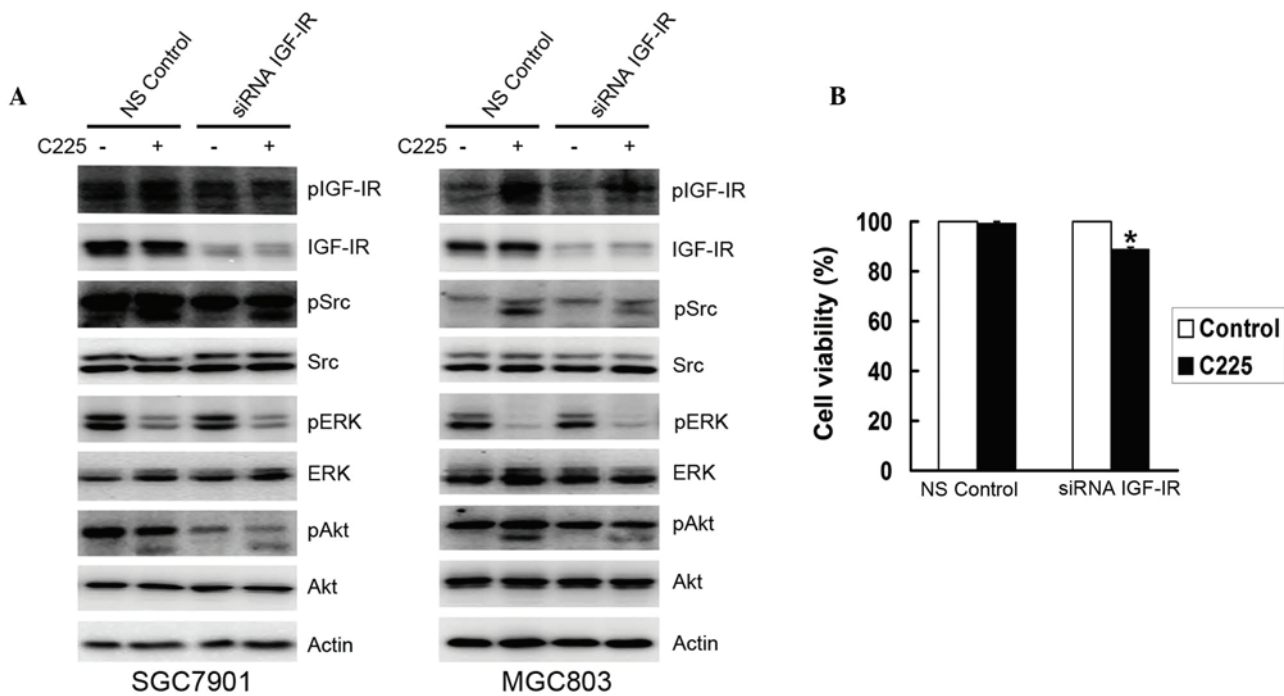


Figure 4. Knockdown of IGF-IR expression reduces cetuximab-induced Src and Akt phosphorylation as well as enhances cellular proliferation inhibition rates in gastric cancer cells. SGC7901 and MGC803 cells were transiently transfected with IGF-IR siRNAs for 48 h, followed by 10 μ g/ml cetuximab for 2 h. (A) Western blot analysis was used to detect the phosphorylation of IGF-IR, Src, ERK and Akt. (B) Cell viability was assessed using an MTT assay. * $P < 0.05$, IGF-IR siRNA cells vs. NS control. IGF-IR, insulin-like growth factor receptor 1; Src, steroid receptor co-activator; ERK, extracellular signal-related kinase; p, phosphorylated; C225, cetuximab; siRNA, small interfering RNA; NS, non-silenced.

MGC803 cells revealed that treatment with cetuximab in combination with OSI-906 produced fewer and smaller colonies than treatment with cetuximab alone (Fig. 3C).

The effect of downregulated IGF-IR gene expression on downstream signaling in gastric cancer cells was examined using IGF-IR-specific siRNAs. As shown in Fig. 4A, western blot analysis was used to confirm the knockdown of IGF-IR. Following exposure to 10 μ g/ml cetuximab for 2 h, IGF-IR-depleted cells exhibited reduced expression of phosphorylated IGF-IR, Src and Akt; however, ERK phosphorylation remained unchanged (Fig. 4A). Cells transfected with IGF-IR siRNAs demonstrated significantly reduced survival rates compared to that of the control cells following exposure to cetuximab for 48 h (Fig. 4B). These results therefore indicated that cetuximab-induced IGF-IR activation was responsible for cetuximab resistance and that Src acted downstream of IGF-IR in gastric cancer cell lines.

Inhibition of Src restores cetuximab sensitivity and represses IGF-IR phosphorylation in gastric cancer cells. In order to investigate the association between Src and IGF-IR, gastric cancer cells were pretreated with the Src inhibitor PP2 (10 μ M) alone or in combination with cetuximab for 2 and 6 h. Activation of IGF-IR was then assessed using western blot analysis. The results revealed that following treatment with PP2, cetuximab-mediated IGF-IR phosphorylation was markedly decreased (Fig. 5A). In addition, gastric cancer cell viability was significantly reduced following cetuximab treatment in combination with PP2 compared to that of cetuximab treatment alone (Fig. 5B). Furthermore, the combination treatment reduced colony formation in MGC803 cells relative to that of treatment with cetuximab alone (Fig. 5C). These results

therefore showed that cetuximab-induced activation of IGF-IR was inhibited following the PP2-induced inhibition of Src activation, indicating that there may be a positive feedback loop between IGF-IR and Src.

Discussion

Numerous studies have confirmed that the primary mechanism of cetuximab resistance was via *K-ras* and *BRAF* gene mutations (36-39). In addition, cetuximab-sensitive gastric cancer cell lines were reported to significantly reduce EGFR activation following cetuximab treatment compared with cetuximab-resistant cells (40). Another study demonstrated that cetuximab failed to inhibit phosphorylation of EGFR pathways in a cetuximab-resistant head and neck squamous cell cancer cell line (41). The results of the present study indicated that cetuximab resistance occurred in gastric cancer SGC7901 and MGC803 cells expressing wild-type *K-ras* and *BRAF*. However, these two cell lines exhibited reduced activation of EGFR and ERK following cetuximab exposure, whereas Akt activation was not affected. It was therefore suggested that other pathways may be involved in Akt activation, thereby mediating cetuximab resistance in gastric cancer cells.

It is widely accepted that EGFR is able to cross-talk with other signaling factors (42-45). A recent study demonstrated that tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was able to activate the EGFR pathway during TRAIL-induced apoptosis in gastric cancer cells (46). Morgillo *et al* (47) reported that the EGFR tyrosine kinase inhibitor erlotinib induced heterodimerization of EGFR/IGF-IR, with activation of IGF-IR and its downstream

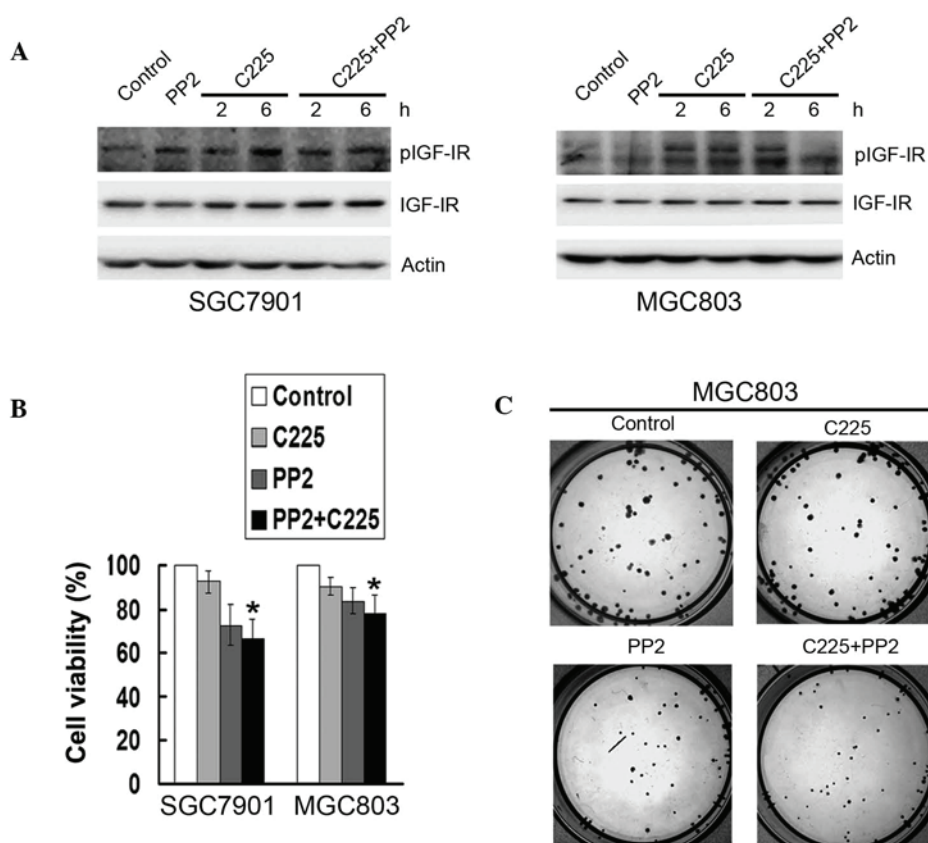


Figure 5. PP2 suppresses IGF-IR phosphorylation and increases the growth inhibitory effect of cetuximab in gastric cancer cells. SGC7901 and MGC803 cells were treated with or without the Src inhibitor PP2 (10 μ M) for 1 h prior to the addition of 10 μ g/ml cetuximab for 2 h and 6 h. (A) Western blot analysis was performed to determine the expression levels of IGF-IR and pIGF-IR. (B) Cell viability was assessed using an MTT assay. * $P < 0.05$, treatment with combination cetuximab and PP2 vs. cetuximab alone. (C) Following 14 days of treatment a colony forming assay was performed. IGF-IR, insulin-like growth factor receptor 1; p, phosphorylated; C225, cetuximab; PP2, 4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-*d*]pyrimidine.

mediator Akt in NSCLC cells; in addition, overexpression of IGF-IR has also been observed in numerous types of human cancers (48) and was shown to be involved in cisplatin resistance (49). Furthermore, it was reported that baseline activation of IGF-IR was correlated with cetuximab resistance (29). The present study found that baseline levels of phosphorylated IGF-IR in gastric cancer cells were not increased; however, following exposure to cetuximab there was a gradual increase in levels of IGF-IR phosphorylation. Furthermore, treatment with the IGF-IR inhibitor OSI-906 or IGF-IR siRNAs inhibited activation of IGF-IR and Akt as well as increased the sensitivity of gastric cancer cells to cetuximab. This therefore indicated that cetuximab-induced IGF-IR and Akt activation were involved in cetuximab resistance in gastric cancer.

In order to further investigate the regulation of cetuximab-induced IGF-IR activation, Src activation was then assessed in the present study. Peterson *et al* (50) reported that IGF-IR was a substrate for v-Src. Src activation was found to occur upstream of IGF-IR transactivation as well as stimulate IGF-dependent proliferation in HEK293 cells and pancreatic carcinoma cells (30,51). By contrast, it was reported that IGF induced Src activation in vascular smooth muscle cells (52). Therefore, the upstream and downstream association between Src and IGF-IR required further elucidation. In the present study, cetuximab was shown to simultaneously induce the activation of IGF-IR and Src. In turn, inhibition of IGF-IR

activation prevented the activation of Src, while inhibition of Src activation inhibited the activation of IGF-IR. This therefore provided evidence for a positive feedback loop between IGF-IR and Src. Furthermore, inhibiting the activation of IGF-IR as well as Src improved gastric cancer-cell sensitivity to cetuximab, therefore indicating that cetuximab induced the activation of IGF-IR and Src, which resulted in cetuximab resistance in SGC7901 and MGC803 gastric cancer cells.

In conclusion, the results of the present study demonstrated that cetuximab blocked EGFR while concurrently inducing activation of IGF-IR and Src. Evidence was provided for a positive feedback loop between Src and IGF-IR, which activated the Akt signaling pathway downstream of EGFR, therefore mediating cetuximab resistance in gastric cancer cells. These present study contributed evidence towards an explanation for the mechanisms underlying cetuximab resistance in gastric cancer cells that retain wild-type *K-ras* and *BRAF* genes. In addition, the results of the present study indicated that inhibition of IGF-IR activation may be an effective mechanism by which cetuximab sensitivity may be enhanced in order to improve the effectiveness of combination chemotherapy in gastric cancer patients.

Acknowledgements

The present study was supported by grants from the Chinese National Foundation of Natural Sciences (nos. 81201802,

81172369, 81172198, 81372485 and 81372546), Specialized Research Fund for the Doctoral Program of Higher Education (nos. 20102104120008 and 20112104110005) and National Science and Technology Major Project (no. 2013ZX09303002).

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