Tumor-penetrating peptide fused EGFR single-domain antibody enhances radiation responses following EGFR inhibition in gastric cancer

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Abstract. Radiotherapy has been the primary method for the local control of several types of unresectable tumor, including gastric cancer. Patients with gastric cancer frequently express high levels of epidermal growth factor receptor (EGFR), which have been found to increase following radiotherapy treatment. This provides a basis for the combination of antibodies targeting EGFR and radiotherapy. In our previous study, a protein (anti-EGFR-iRGD) with bispecific targets and high permeability was constructed, and its effects on inhibiting the proliferation of gastric cancer cells was investigated. In the present study, the capacity of anti-EGFR-iRGD to modulate a radiation response was investigated and the specific mechanisms underlying these interactions were evaluated in gastric cancer cell lines and xenografts exhibiting high levels of EGFR. The radioenhancement of anti-EGFR-iRGD was associated with inhibited radiation-induced upregulation of EGFR, inhibited cell proliferation and promotion of cell apoptosis. In addition, anti-EGFR-iRGD appeared to permeate more into the tumor tissue following radiation. These findings indicated that the recombinant protein anti-EGFR-iRGD was a selective and effective radiosensitizer in EGFR-overexpressing gastric cancer cells and xenografts. These results further suggested that anti-EGFR-iRGD is a potential superior EGFR-targeted therapy combined with radiotherapy. Overall, the present study suggested that anti-EGFR-iRGD may be a promising candidate for preclinical and clinical use.

Introduction

Gastric cancer is the fourth most common malignant tumor worldwide and the second leading cause of cancer-associated mortality (1), 70% of which occur in developing regions, including 40% of individuals in China (2). Despite advances in cancer therapy, the majority of advanced malignancies remain incurable. Radiotherapy is the main method of local control for several types of unresectable tumor, and for controlling gastric bleeding. Previous studies have shown that radiotherapy did not improve the survival rate of patients with gastric cancer, whereas local control rates were 70% (3). Radiotherapy is considered an attractive modality for the high incidence of locoregional failures following surgical treatment of gastric cancer (4,5).

Tumors in humans frequently express high levels of epidermal growth factor receptor (EGFR), which has been associated with poor prognosis when expressed at high levels (6). In 511 cases of gastric carcinoma, the expression of EGFR was 27.4% (7). In several cases, including gastric cancer, the overexpression of EGFR drives tumor cells towards uncontrolled proliferation, allowing the cells to evade programmed death, thereby enhancing their ability to migrate and metastasize. The activation of EGFR is involved in the resistance of tumor cells to radiotherapy (8). In response to radiation, EGFR is rapidly activated and induces several
downstream signaling pathways, including mitogen-activated protein kinase (MAPK)-extracellular signal regulated kinase (ERK) and phosphoinositide 3-kinase (PI3K)/Akt. Activation of these signaling pathways may promote cell proliferation and apoptosis avoidance, and the repair of radiation-induced DNA damage through homologous and non-homologous recombination (9). Repeated exposure to radiation also results in increased expression of EGFR (9,10). Therefore, EGFR inhibitors are the most promising molecular targeting agents for use in combination with radiotherapy (11-13). Advances in the field of genetic engineering have led to the development of various EGFR inhibitors, including monoclonal antibodies, tyrosine kinase inhibitors (TKIs), antisense oligonucleotides and single-domain antibody (14). In a previous study, the application of cetuximab during primary radiotherapy in patients with head and neck squamous cell carcinoma resulted in improved locoregional tumor control and survival rates compared with patients who received radiotherapy alone (11). These pioneering findings have paved the way for the clinical use of EGFR inhibitors in combination with radiotherapy.

In our previous study, a tumor-penetrating peptide was constructed that was fused with an EGFR single-domain antibody (15), termed anti-EGFR-iRGD, which consisted of an anti-EGFR VHH, from the variable domain of the heavy chain of the antibody, fused to iRGD. The tumor specific binding peptide exhibited high permeability into the tumor. In addition, the recombinant protein anti-EGFR-iRGD showed antitumor activity in tumor cell lines, multicellular spheroids and mice (16). Radiotherapy is widely used in the treatment of various types of cancer. In the present study, the effects of anti-EGFR-iRGD treatment in combination with radiotherapy were investigated in gastric cancer with high levels of EGFR.

Materials and methods

Cell culture, xenograft experiments and ionizing radiation. Three human gastric adenocarcinoma cell lines (SNU-719, BGC-823 and HGC-27) were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen; Grand Island, NY, USA) supplemented with 10% bovine calf serum (BCS; Life Technologies/Gibco, Grand Island, NY) in 5% CO₂ at 37°C. All animal procedures were performed in compliance with the guidelines set by the Animal Care Committee at Drum Tower Hospital (Nanjing, China). A total of 5,000,000 BGC-823 gastric cancer cells in 0.1 ml of PBS were subcutaneously injected in the lower right flank of athymic nude BALB/c mice (5-6 weeks old, female, 18-22 g, Shanghai Experimental Animal Center, Shanghai, China). BALB/c mice were kept in climate-controlled quarters with a 12-h light and dark cycle with food and water in cages under germ-free conditions. Tumor volumes were calculated from two diameter measurements according to the following formula: Tumor volume = (length x width²)/2. Radiotherapy was administered in vitro using a 6 MeV X-ray linear accelerator (Elekta AB, Stockholm, Sweden).

Cell viability assay and flow cytometry assays. Following treatment with anti-EGFR-iRGD, cell viability was evaluated using an MTT assay. In brief, the cells were seeded into 96-well plates at a density of 3,000-8,000 cells/well. Subsequently, cells in the logarithmic phase were treated with anti-EGFR-iRGD at indicated concentrations (6.3, 12.5, 25, 50, 100, 200, 400 and 800 µg/ml). Following incubation for 24 h at 37°C, MTT reagent was added, followed by dimethyl sulfoxide (DMSO), and the spectrophotometric absorbance was measured (490 nm). To detect apoptosis, cells in the logarithmic phase were treated with anti-EGFR-iRGD for 24 h 37°C. The cells were harvested, washed with PBS, and subsequently incubated in the dark for 15 min at room temperature. Finally, the degree of apoptosis was analyzed by FACScan laser flow cytometry (BD Aria II; BD Biosciences, Franklin Lakes, NJ, USA) using an FITC Annexin V Apoptosis Detection kit (Roche Applied Science, Indianapolis, IN, USA). The number of cells analyzed for each sample was 50,000.

Clonogenic survival assay. The cells were seeded into 6-well plates at a density of 500-8,000 cells per well. Following incubation for 24 h, anti-EGFR-iRGD (100 µg/ml) was added into each well. The cells were treated with anti-EGFR-iRGD for 24 h at 37°C and then exposed to increasing doses of ionizing radiation (0, 2, 4, 6 and 8 Gy). Following intervals of 7-10 days, cell colonies (consisting of ≥50 cells) were stained with crystal violet and counted manually using optical microscopy.

Western blot assay. The expression levels of EGFR in gastric cancer cells were confirmed by western blot analysis. Cell lysates were prepared with a detergent buffer, as previously described (17). Protein concentrations were measured with the BCA Protein Assay according to the manufacturer's manual (Beyotime Institute of Biotechnology, Shanghai, China). The proteins (30 µg) were separated by 10% SDS-PAGE, and transferred onto polyvinylidylen difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA; Life Technologies/Gibco) in Tris-buffered saline, containing 0.05% Tween-20 for 2 h at room temperature, and then incubated overnight at 4°C with a 1:2,000 dilution of primary antibody targeting EGFR (dilution 1:2,000; cat. no. 4267; Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (dilution 1:2,000; cat. no. AF0003; Beyotime Institute of Biotechnology, Haimen, China). The membranes were incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse (1:2,000; cat. no. A0216; Beyotime Institute of Biotechnology) and goat anti-rabbit antibodies (dilution 1:2,000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at room temperature and detected by ECL reagents (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Penetration in tumor tissue. Following radiation, the distribution of anti-EGFR-iRGD in tumor tissues was determined by laser scanning confocal microscopy (LSCM). Animal models were used to locate proteins and the permeability of recombinant proteins following radiotherapy was examined. The BALB/c mice (n=3 mice per group) were subcutaneously injected with EGFR-overexpressing BGC-823 cells (5,000,000 gastric cancer cells in 0.1 ml of PBS), in the right flank (no radiation, 0 GY) and in the left flank (radiation, single dose of 2 Gy). When the tumors reached a volume of ~150 mm³, radiotherapy was delivered to the left flank at 600 cGy/min with 6 MV X-rays. The mice received a
single dose of 2 Gy. At 24 h post-radiation treatment, rhodamine-B-labeled anti-EGFR-iRGD was administered to the BGC-823 tumor-bearing mice via tail vein injection. The mice were sacrificed and tumors were harvested 1 h following the administration of anti-EGFR-iRGD. The tumors were frozen and sections were cut. Finally, the tumor sections (5 µm) were subjected to DAPI staining and visualized using LSCM.

**In vivo antitumor effect.** The gastric cancer cells (BGC-823) were subcutaneously injected into BALB/c mice. When the subcutaneous tumor was ~100 mm³, the mice were randomly divided into four groups. The day of randomization was designated as ‘Day 1’. The mice were treated every day by intraperitoneal injection with anti-EGFR-iRGD at 1 mg on the first day (day 1) and either 0.6 mg during each subsequent injection (a total of five injections). At 24 h following the first injection, radiotherapy was delivered to one field, including the tumor, with 5-mm margins, using a Clinac 2300 C/D linear accelerator. Radiation was delivered at 600 cGy/min with 6 MV X-rays beams at doses of 10 Gy, in five fractions, with one fraction each day. The mice were monitored daily, and tumor volume and body weight were recorded every 3 days. The mice were sacrificed at the end of the experiment. Following treatment, histological observation of the heart, liver, spleen, lung and kidney, and tumor tissues was performed.

**Immunostaining of tumor sections and organs.** The xenografts and organs were fixed in neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for pathological observation. The tissues were sectioned at a thickness of 5 µm and the sections were evaluated using optical microscopy.

**Statistical analysis.** SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) was used for statistical analysis. T-test was used to compare the means between two groups, where their variances of both groups may be different. One-way analysis of variance was used for multiple comparisons. Covariance analysis was used for comparison between four groups to remove the effects of the covariate. P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation.

**Results**

**In vitro cytotoxicity of recombinant protein anti-EGFR-iRGD and expression of EGFR in gastric cancer cell lines.** In vitro cytotoxicity was assessed using MTT assays, which showed that, even at a low concentration, anti-EGFR-iRGD exhibited anti-proliferative activity against the SNU-719 cells and BGC-823 cells. Furthermore, a dose-dependent effect of anti-EGFR-iRGD was observed in the SNU-719 cells and BGC-823 cells. However, in the HGC-27 cells (no EGFR expression), no anti-proliferative activity was observed, even at the highest concentration of 800 µg/ml (Fig. 1A). To select appropriate cell lines as the study objective and to investigate the expression levels of EGFR in different gastric cancer cell lines, the three human gastric cancer cell lines (SNU-719, BGC-823 and HGC-27) were evaluated by western blot analysis. The data revealed the following descending expression levels of EGFR: BGC-823>SNU-719>HGC-27 (Fig. 1B). These findings indicated that the anti-proliferative activity of anti-EGFR-iRGD in human gastric cancer cells was associated with the expression of EGFR.
Anti-EGFR-iRGD modulates radiosensitivity. To evaluate the potential capacity of combining anti-EGFR-iRGD with radiation in human gastric cancer cells, experiments were performed to examine the effect of anti-EGFR-iRGD on clonogenic survival. The clonogenic survival curves of SNU-719, BGC-823 and HGC-27 cells are shown in Fig. 1C, in which cells were exposed to anti-EGFR-iRGD and radiation (anti-EGFR-iRGD prior to radiation). The data indicated that treatment with anti-EGFR-iRGD prior to radiation induced modest but consistent radiosensitization, as manifested by a reduction in clonogenic survival compared with control exposure to ionizing radiation alone, in SNU-719 and BGC-823 cells treated with 2, 4, 6 and 8 Gy.

Anti-EGFR-iRGD enhances radiation-induced apoptosis. To investigate the inhibitory effects of the combination treatment of anti-EGFR-iRGD ± radiation on cell growth, the apoptotic responses of recombinant proteins combined with ionizing radiation (single dose of 6 Gy) were investigated in SNU-719, BGC-823 and HGC-27 gastric cancer cells. Q2 represents early apoptotic cells, Q2+Q4 indicates gastric cells that are not viable. Compared with the cells that received ionizing radiation alone or recombinant protein alone, the SNU-719 cells (Fig. 2A) and BGC-823 cells (Fig. 2B) pretreated with anti-EGFR-iRGD protein showed a significant increase in apoptosis (P<0.0001). However, compared with the cells that received ionizing radiation alone, the HGC-27 cells (Fig. 2C) pretreated with anti-EGFR-iRGD protein did not show an increase in apoptosis (P>0.05). These findings indicated that the recombinant protein-enhanced apoptosis of human gastric cancer cells was associated with the expression of EGFR.

Mechanism of recombinant protein enhances the radiation response. Following ionizing radiation for 24 h, the expression of EGFR in tumor tissue sections was analyzed. The expression pattern revealed mainly membrane-bound EGFR staining, indicating that, following ionizing radiation, EGFR was upregulated (Fig. 3A). As shown in Fig. 3B, the increased upregulation of EGFR was confirmed following radiation exposure (single dose of 2 Gy) in the BGC-823 cell lines. The radiation-induced upregulation of EGFR was inhibited by pretreatment of the tumor cells with 100 µg/ml anti-EGFR-iRGD for 24 h. These findings showed that anti-EGFR-iRGD treatment combined with radiotherapy effectively inhibited the expression of EGFR in EGFR-overexpressing gastric cancer cells.

Evaluation of the penetration of anti-EGFR-iRGD into tumor tissue following radiotherapy. BALB/c mice were subcutaneously injected with BGC-823 cells, in the right flank (no radiation, 0 Gy) and in the left flank (radiation, 2 Gy). The penetration ability of recombinant protein anti-EGFR-iRGD following radiotherapy was then analyzed with tumor tissue sections derived from BGC-823-bearing mice. The penetration of anti-EGFR-iRGD was also evaluated in BGC-823 tumors at 1 h post-injection and following radiation (2 Gy) for 24 h. Rhodamine B-labeled proteins (red) and DAPI-labeled nuclei (blue) were present in the images of tumor sections. Following radiation with 2 Gy for 24 h, the penetration...
of anti-EGFR-iRGD into the tumor tissues had increased (Fig. 3C).

**Anti-EGFR-iRGD augments the in vivo tumor response of gastric cancer xenografts to radiation.** The in vivo activity of anti-EGFR-iRGD ± radiation in tumor xenografts was examined. BGC-823 (5×10⁶) cells were injected subcutaneously into the flank of athymic mice. The mice were treated with PBS (control) or anti-EGFR-iRGD (1.0 mg on the first day, 0.6 mg every day from day 2-5 via intraperitoneal injection), ionizing radiation (2.0 Gy/fraction; five fractions/week; total of five fractions), or a combination of anti-EGFR-iRGD and ionizing radiation. As shown in Fig. 4A, treatment with radiation alone or anti-EGFR-iRGD alone produced modest inhibition of tumor growth in BGC-823 xenografts. However, when combined with radiation, anti-EGFR-iRGD enhanced the tumor growth inhibition profile over the 24-day observation period.

**In vivo expression of proliferating cellular nuclear antigen, apoptosis and necrosis.** BGC-823 tumor xenografts were used for evaluation of the expression of markers of tumor proliferation (Ki-67). The immunohistochemical staining for Ki-67 indicated that the number of proliferating cells were the lowest in the combined treatment group, intermediate in the groups receiving single modality treatment with either anti-EGFR-iRGD or radiation, and highest in the control group. Furthermore, TUNEL staining results showed that the number of apoptotic cells in the combined treatment group was marginally higher than that in the radiotherapy group and the fusion protein group. However, there was no statistically significant difference between three treated groups because the F statistic of one-way ANOVA was 1.679 with P=0.227>0.05 (The F statistic of the variance homogeneity test between tree groups was 0.344 with P=0.716>0.05). The pathological examination showed tumor necrosis in all treatment groups. However, the necrotic area of the PBS-treated control group was the smallest, whereas the anti-EGFR-iRGD and radiation-treated groups had larger necrotic regions. The largest necrotic regions were apparent in the combined treatment group. These results demonstrated the capacity of anti-EGFR-iRGD in modulating cellular proliferation and cell necrosis (Fig. 4B).

**Side effects of anti-EGFR-iRGD with ionizing radiation.** As shown in Fig. 5A, none of the mice treated with
anti-EGFR-iRGD, ionizing radiation or combination treatment showed any body weight loss. The mean body weights of mice in the three treatment groups were marginally lower than that of mice in the control group, however, no significant differences were observed in body weight between the four groups by covariance analysis (P=0.174>0.05). The H&E staining of the organs (Fig. 5B) showed that tissue changes comprised only the presence of inflammatory cells that infiltrated the spleen.
inhibition of EGFR may protect cells in the first few hours following radiation, whereas the combined effects of GI arrest and DNA repair inhibition may result in increased sensitization 24 h following inhibition.

Although EGFR has often been described as a cell surface receptor, it is closely associated with several nuclear processes. In addition, resistance to radiation has been associated with nuclear levels of EGFR (38). Nuclear EGFR signaling is important in gene regulation, but also affects DNA repair. Nuclear EGFR is involved in resistance to EGFR-targeted therapies. In addition to the classic mechanism of DNA damage, high dose per fraction radiation (>8 Gy) may generate stromal effects that are not accounted for in traditional radiobiological modeling (41). Anti-EGFR-iRGD, which specifically targets EGFR, spreads extensively throughout the tumor mass. Furthermore, following radiation for 24 h, anti-EGFR-iRGD appear to permeate even further into the tumor tissue. The combination of an increased degree and/or different modes of DNA damage and injury to the tumor microenvironment arising from the use of hypofractionation may work synergistically to cause irreparable and lethal injuries to irradiated tumor cells (41,42).

For EGFR-targeted therapies to be successful, appropriate patient selection is required to optimize efficacy. The RTOG 0617 study showed the importance of patient selection when EGFR-targeted therapy with radiotherapy was used (43). Significant progress has been made in the development of novel radiation approaches. However, the integration of targeted therapy and radiotherapy has raised several unresolved questions, including the identification of patients, optimal dose and time of radiation, treatment sequence, and side effects of treatment. Therefore, further investigations are required to better analyze targeted therapies and, in particular, the combination of antibodies and radiotherapy.

In conclusion, given the importance of EGFR in several types of cancer and the well-defined role of EGFR in the response to radiotherapy, this receptor is an important target when treatment is combined with radiotherapy. The present study demonstrated that anti-EGFR-iRGD was an effective radiosensitizer in EGFR-overexpressing gastric cancer cells and xenografts. The radioenhancement in gastric cancer cells and xenografts was associated with inhibited radiation-induced upregulation of EGFR, inhibited cell proliferation and promotion of cell apoptosis. In conclusion, anti-EGFR-iRGD was a selective and effective radiosensitizer in gastric cancer, which makes it a potential superior EGFR-targeted therapy for further preclinical and clinical use.

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Availability of data and materials
The datasets analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions
BL, QL, HS and FJ conceived and designed the study. FM, AZ, ND, HZ, HQ and LY were involved in the experiments and drafted the manuscript. HX performed the statistical analysis. BL, QL, HS and FJ wrote and revised the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate
The study protocol was reviewed and approved by the Ethics Committee of Nanjing Drum Tower Hospital, The Affiliated Hospital of Nanjing University Medical School.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


