A ligand-based and enediyne-energized bispecific fusion protein targeting epidermal growth factor receptor and insulin-like growth factor-1 receptor shows potent antitumor efficacy against esophageal cancer

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Abstract. Recent studies have revealed that the epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R) are overexpressed in various types of human tumors and are attractive targets for anticancer drugs. In the present study, the expression of EGFR and IGF-1R in esophageal squamous cell carcinoma (ESCC) and adjacent normal tissues in a tissue microarray was firstly detected by immunohistochemical staining. In addition, their co-overexpression was observed in 48 out of 75 (64%) patients. Based on the findings, the antitumor activity of an EGFR/IGF-1R bispecific and enediyne-energized fusion protein EGF-LDP-IGF-AE, which we constructed recently by fusing two ligands (EGF and IGF-1) with an enediyne antibiotic lidamycin (LDM), on ESCC were evaluated. Binding assay indicated that the EGF-LDP-IGF protein bound to esophageal cancer cells, and then internalized into the cytoplasm. *In vitro*, the enediyne-energized fusion protein EGF-LDP-IGF-AE exhibited extremely potent cytotoxicity to ESCC cells with IC_{50} values between 10^{-10} and 10^{-15} mol/l. *In vivo*, EGF-LDP-IGF-AE also markedly suppressed the growth of human KYSE450 xenografts by 75.1% when administered at 0.3 mg/kg in a nude mouse model, and its efficacy was significantly higher than that of LDM (at maximum tolerated dosage) and mono-specific counterparts. In addition, EGF-LDP-IGF-AE arrested cell cycle progression and it concentration-dependently induced cell apoptosis as well as inhibited the activation of EGFR/IGF-1R and two major downstream signaling pathways (PI3K/AKT and RAS/MAPK). These data imply the potential clinical application of EGF-LDP-IGF-AE for ESCC patients with EGFR and/or IGF-1R overexpression.

Introduction

The morbidity and mortality of esophageal cancer rank the eighth and sixth among all malignant tumors worldwide (1). Esophageal cancer is classified into esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (EAC) based on the histopathologic type. In Western countries, EAC represents the dominant subtype and the incidence has increased markedly over the past decades, whereas the northern regions of Henan Province, China, have the highest incidence of ESCC (2). Despite the great advances in early diagnosis and traditional treatment options (surgery, chemotherapy and radiotherapy), the prognosis of patients with advanced esophageal cancer remains poor with the 5-year survival rate ranging from 15 to 25% (3). During the past decade, the field of drug development has been transformed with the identification of and ability to direct treatment at specific molecular targets. The overexpression and aberrant function of epidermal growth factor receptor (EGFR) and insulin-like growth factor-1 receptor (IGF-1R) in a number of solid tumors including esophageal cancer, and the important roles in the development of tumors have provided a rationale for targeting the two receptors.

EGFR/HER1 is a member of the ErbB receptor tyrosine kinase family, and there are three other members, HER2, HER3 and HER4, in this family. Ligand (EGF and TGF-α) binding to the receptors results in receptor homodimerization and heterodimerization, activation of the intrinsic kinase domain and initiation of a cascade of downstream signaling that ultimately promotes tumor cell survival, proliferation, invasion and metastasis (4). EGFR overexpression has been observed in many human tumors, such as lung, head and neck,

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colorectal, breast and ovarian (5-9). In esophageal cancer, ~50-71% of ESCC patients have EGFR overexpression (10). The IGF system is comprised of the IGF ligands (IGF-1 and IGF-2), cell-surface receptors (IGF-1R and IGF-2R) and six IGF-binding proteins (IGFBPs). IGF-1R is a tetrameric glycoprotein containing two α subunits and two β subunits linked by disulfide bonds, and there is a 60% homology between IGF-1R and the insulin receptor. IGF-1R binds IGF-1 with high affinity and IGF-2 and insulin with a lower affinity. Similarly, when the ligands bind to IGF-1R, auto-phosphorylation of the receptor tyrosine kinase is induced, leading to the activation of multiple downstream signaling pathways, such as PI3K/AKT and RAS/MAPK, each of which plays an important role in cell proliferation, migration and metabolism. IGF-1R overexpression has also been found in many tumors, including 60% of esophageal cancer, and its overexpression was found to correlate with advanced tumor stage, depth of invasion, metastasis and recurrence (11,12). IGF-1R and EGFR families show homology in their structure and both the receptors share considerable crosstalk in their functions. Co-expression of the two receptors was observed in resected non-small cell lung cancer (NSCLC), colorectal cancer, adrenocortical carcinoma, pancreatic ductal adenocarcinoma, and was found to be associated with poor pathological stage and shorter disease-free survival (DFS) (13-16). A number of EGFR-targeted drugs (gefitinib, erlotinib, cetuximab and panitumumab) alone or in combination with chemotherapeutics have been tested clinically for the treatment of patients with esophageal cancer, but the results are disappointing (17-21). Different from the monoclonal antibodies and tyrosine kinase inhibitors, antibody-drug conjugates or ligand-toxin fusion proteins taking advantage of the specificity of antibodies or ligands and the potent cytotoxic activity of toxins have shown elevated antitumor efficacy. Furthermore, they are effective even for patients who are resistant to targeted drugs. For example, trastuzumab emtansine (T-DM1) consisting of trastuzumab coupled to a cytotoxic agent, emtansine (DM1), showed significantly improved progression-free and overall survival with less toxicity than lapatinib plus capecitabine in patients with HER2-positive advanced breast cancer previously treated with trastuzumab and a taxane (22).

EGF-LDP-IGF-AE is a bispecific and enediyne-energized fusion protein targeting both EGFR and IGF-1R that was previously constructed by us (23). It contains two ligands (EGF and IGF-1) specific for EGFR and IGF-1R and an enediyne antibiotic lidamycin (LDM) with potent cytotoxicity. The two ligands were designed as targeting moiety, directing the fusion protein to cancer cells with EGFR and IGF-1R overexpression. LDM is composed of a noncovalently bound apoprotein (LDP) and an active enediyne chromophore (AE) that act as a cytotoxic moiety as previous studies have demonstrated the extremely potent cytotoxicity of LDM to various tumor cells and marked inhibitory effects on a panel of xenografts in athymic mice (24). In the present study, we measured the *in vitro* and *in vivo* antitumor activity of EGF-LDP-IGF-AE on esophageal cancer and the efficacy was compared with corresponding mono-specific fusion proteins to determine whether dual inhibition of EGFR and IGF-1R is more efficacious. The underlying mechanisms of the antitumor effect of EGF-LDP-IGF-AE were also evaluated.

Materials and methods

Ethics statement. All experiments involved in animals were performed according to the Declaration of Helsinki and according to international and national guidelines, and the procedures were approved by the Ethics Committee of Xinxiang Medical University.

Tissue microarray and immunohistochemical staining. Tissue microarrays containing a total of 75 pairs of human ESCC tumor and corresponding adjacent normal tissues (HEso-Squ150CS-01), were purchased from Shanghai Outdo Biotech Co. Ltd. (Shanghai, China), and the immunohistochemical (IHC) staining was used to analyze EGFR and IGF-1R expression. Tissue microarray slides were deparaffinized in xylene, rehydrated with graded ethanol and immersed in water. For antigen retrieval, the slides were heated at 95˚C for 40 min and incubated with 3% hydrogen peroxide at room temperature for 15 min. Mouse anti-EGFR or anti-IGF-1R antibody (diluted 1:100; Lab Vision Corporation, Fremont, CA, USA) was applied overnight at 4˚C, followed by Polymer Helper (ZSGB-Bio, Beijing, China) for 20 min. Subsequently, the slides were incubated with polyperoxidase anti-mouse IgG (ZSGB-Bio) at 37˚C for 30 min. According to the manufacturer's instructions, the slides were reacted with DAB liquid system (Dako, Glostrup, Denmark) and counterstained with hematoxylin. The assessment of EGFR or IGF-1R staining was performed by two pathologists separately using H-score systems as previously described (25): scores ≥201, 101-200, 1-100, 0 represent strongly positive (3+), moderately positive (2+), weakly positive (1+) and negative (-) staining, respectively.

Cell lines and culture conditions. Human ESCC cell lines EC9706, TE-1, KYSE450 and KYSE510 were obtained from the Cell Center of Peking Union Medical College (Beijing, China). Cells were cultured in RPMI-1640 medium (Gibco; Life Technologies) containing 10% (v/v) fetal bovine serum (FBS; Gibco, Life Technologies) and 100 μg/ml streptomycin, 100 U/ml penicillin at 37˚C with 5% CO_2.

Preparation of fusion proteins and their enediyne-energized analogues. DNA sequences coding for fusion protein EGF-LDP-IGF, which contains the gene of human EGF (169 bp), $(G_4S)_2$-linker (30 bp), apoprotein of LDM (ldp, 330 bp), $(G_4S)_2$-linker (30 bp) and human IGF-1 (210 bp) from 5' to 3' end, were synthesized by Beijing Sunbiotech Co. Ltd. (Beijing, China), and then, it was cloned into the pET30a vector to generate the plasmid pET30-*egf-ldp-igf*. EGF-LDP-IGF protein was expressed in the *Escherichia coli* strain BL21(DE3) according to the pET System Manual (11th edition; Novagen, Madison, WI, USA) and purified by Ni^{2+} affinity chromatography (HisTrap HP column; GE Healthcare, Milwaukee, WI, USA), since the His_6-tag was introduced to the COOH terminal of EGF-LDP-IGF protein. The active chromophore of LDM (AE) was isolated using a C4 column (GE Healthcare) with 22% acetonitrile in 0.05% trifluoro-actic acid mobile phase, and then the enediyne-energized analogue of fusion protein EGF-LDP-IGF-AE was prepared by integrating the AE into EGF-LDP-IGF. The corresponding mono-specific fusion proteins (EGF-LDP and LDP-IGF)

and their enediyne-energized analogues (EGF-LDP-AE and LDP-IGF-AE) were constructed in the same way.

Binding affinity assay. The binding affinity of EGF-LDP-IGF protein to esophageal cancer cells was analyzed by immuno-fluorescence staining assay. Cells were grown on coverslips, cultured for 24 h and fixed with 4% paraformaldehyde for 10 min at room temperature. After washed three times with 0.05% Tween-20 in phosphate-buffered saline (PBS) for 5 min each, the cells were blocked with 5% bovine serum albumin (BSA; Genview, China) for 1 h and subsequently incubated with EGF-LDP-IGF protein (50 μg/ml) for 2 h at room temperature. Then, they were incubated with mouse anti-His-tag antibody (diluted 1:100; Tiangen Biotech, China) overnight at 4°C, washed for three times, followed by Alexa Flour 488-labeled goat anti-mouse antibody (diluted 1:50; Beyotime Biotechnology, Shanghai, China) for 1 h. After being washed five times with PBS, the cells were stained with Hoechst 33258 (Beyotime Biotechnology) for 15 min at room temperature. The images were observed under a Zeiss LSM 780 confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

Cell viability assay. MTT assays were used to measure the cytotoxicity of enediyne-energized fusion proteins to esophageal cancer cells *in vitro*. Cells were seeded in 96-well plates (1,000-2,000 cells/well) and incubated for 24 h at 37°C with 5% CO_2. LDM and enediyne-energized fusion proteins (EGF-LDP-IGF-AE, EGF-LDP-AE and LDP-IGF-AE) at different concentrations were added to each well for 48 h of incubation. Then, 20 μl MTT (5 mg/ml; Sigma) was subsequently added and incubated for another 4 h. The supernatant was removed and 150 μl dimethyl sulfoxide (DMSO) was added to each well. The absorbance at 570 nm was measured by an ELISA reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Growth inhibition was calculated as the percentage of the untreated controls and the IC_{50} values were calculated by GraphPad Prism 5.

Cell cycle distribution analysis. Propidium iodide (PI) staining was used for evaluating the effects of bispecific fusion protein EGF-LDP-IGF-AE on cell cycle distribution. Cells ($2x10^4$) were plated in 60-mm dishes, cultured for 24 h and treated with 0.01, 0.05 and 0.1 nmol/l EGF-LDP-IGF-AE for 48 h. Subsequently, the cells were digested by trypsin-EDTA and fixed with cold 70% ethanol. After being washed three times with PBS, the cells were resuspended in 500 μl staining buffer containing PI (50 mg/ml, 25 μl) and RNase A (100 mg/ml, 10 μl) and incubated at 37°C for 30 min according to the manufacturer's instructions (Beyotime Biotechnology). Then, cells were analyzed for fluorescence with a flow cytometer (BD Biosciences, Heidelberg, Germany).

Cell apoptosis assay. The effect of EGF-LDP-IGF-AE on the apoptosis of esophageal cancer cells was investigated by Annexin V-FITC/PI staining. Cells were cultured in 6-well plates for 24 h and treated with 0.1, 0.5, 1 and 2 nmol/l of EGF-LDP-IGF-AE for 48 h. Cells were harvested, washed twice with PBS, resuspended in 500 μl binding buffer containing 10 μl Annexin V-FITC and 5 μl PI (Beyotime Biotechnology), incubated at room temperature for 10 min, and analyzed for fluorescence with a flow cytometer (BD Biosciences).

Western blot analysis. KYSE450 or EC9706 cells were seeded in 100-mm dishes and grown to 70-80% confluence, after which the cells were washed twice in PBS and cultured overnight in serum-free medium. Cells were firstly exposed to EGF-LDP-IGF-AE (0.1 nmol/l), EGF-LDP-AE (0.1 nmol/l) or LDP-IGF-AE (0.1 nmol/l) for 24, 48, 72 or 96 h, followed by stimulation with human EGF (50 ng/ml), human IGF-1 (50 ng/ml) (both from Abcam, Cambridge, MA, USA), or both for 30 min at 37°C. Cells were then collected and lysed in cell lysis buffer (Beyotime Biotechnology) containing 1 mmol/l phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min. Total proteins (30 μg) extracted from the cells were applied on 10% SDS-PAGE and transferred to polyvinylidenedifluoride membranes (PVDF; Millipore, Billerica, MA, USA). After being blocked with 5% BSA for 1 h at room temperature, the membranes were incubated with primary antibodies (diluted 1:1,000) overnight at 4°C and secondary HRP-conjugated anti-bodies (diluted 1:4,000) (both from Cell Signaling Technology, Beverly, MA, USA) for 1 h after being washed three times with 1X TBST buffer. The specific bands were visualized with the Immobilon Western Chemiluminescent HRP Substrate kit (Millipore) and captured by Amersham Imager 600 system (GE Healthcare, Logan, UT, USA).

In vivo efficacy assay. Female BALB/c nude mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China), and the KYSE450 xenograft nude mouse model was performed to evaluate the *in vivo* efficacy of fusion proteins. KYSE450 cells ($5x10^7$) suspended in 200 μl PBS were inoculated s.c. in the right armpit of nude mice. When the tumor size was >100 mm^3, the nude mice were randomly divided into six groups (n=6) and treated with EGF-LDP-AE (0.3 mg/kg), LDP-IGF-AE (0.3 mg/kg), EGF-LDP-IGF-AE (0.2 and 0.3 mg/kg) and LDM (0.05 mg/kg), respectively. They received a 200 μl volume of PBS and injected i.v. in the tail vein. Ten days after the first treatment, tumor-bearing mice were injected with the fusion proteins again at the same doses. Tumor size was measured every third day and tumor volume (V) was determined using the formula: V = length x width^2/2. The inhibition rates were calculated using the formula: 1 - tumor volume (treated)/tumor volume (control) x 100%.

Statistical analysis. Results of the present study were derived from three independent experiments, analyzed by GraphPad Prism 5 software, and are presented as mean ± SD. One-way ANOVA or two-way ANOVA and Bonferroni post hoc analysis were used to compare the differences between groups. P-values <0.05 were considered as statistically significant. The densitometry analysis of the western blot results was analyzed by ImageJ software.

Results

EGFR and IGF-1R are overexpressed in esophageal cancer tissues. As shown in Fig. 1A and B, there was strong and specific expression of both EGFR and IGF-1R in the ESCC

Table I. EGFR and IGF-1R expression in esophageal squamous cell carcinoma and paired normal esophageal tissues in a tissue microarray.

Tissue	EGFR expression	n (%)	IGF-1R expression	n (%)
Esophageal squamous cell carcinoma	Negative	16 (21.3)[a]	Negative	13 (17.3)[b]
	Positive	59 (78.7)[a]	Positive	62 (82.7)[b]
	Low (1+)	47 (62.7)	Low (1+)	39 (50.7)
	Medium (2+)	12 (16.0)	Medium (2+)	17 (24.0)
	High (3+)	0 (0.0)	High (3+)	6 (8.0)
Paired normal esophageal epithelium	Negative	48 (64)[a]	Negative	38 (55.1)[b]
	Positive	27 (36)[a]	Positive	31 (44.9)[b]
	Low (1+)	26 (33.4)	Low (1+)	26 (37.7)
	Medium (2+)	1 (1.3)	Medium (2+)	5 (7.2)
	High (3+)	0 (0.0)	High (3+)	0 (0.0)

EGFR and IGF-1R expression levels were evaluated by H-score test after immunohistochemical staining, and for H-score test: negative=0, low=1-100, medium=101-200, high ≥201. The samples of paired normal esophageal epithelium used for IGF-1R expression were 69 due to the tissue detachment in six samples. [a]$P<0.0001$, the difference between EGFR-positive and -negative cases between esophageal squamous cell carcinoma and normal esophageal epithelium was analyzed for significance by Chi-square test. [b]$P<0.0001$, the difference in IGF-1R-positive and -negative cases between esophageal squamous cell carcinoma and normal esophageal epithelium was analyzed for significance using Chi-square test. EGFR, epidermal growth factor receptor; IGF-1R, insulin-like growth factor-1 receptor.

tissues. The negative and positive cases for EGFR in ESCC tissues were 16 and 59, respectively, which were significantly different from the paired adjacent normal tissues (the negative and positive cases of 48 and 27, respectively; Chi-square test, $P<0.0001$). The expression of IGF-1R in the ESCC and paired adjacent normal tissues was also significantly different (Chi-square test, $P<0.0001$; Table I). The EGFR and IGF-1R expression results from the tissue microarray are summarized in Table I, and representative examples of negative and positive staining with intensities of 1+, 2+ and 3+ are presented in Fig. 1C. EGFR expression was positive in 78.67% of the tumor tissues (59/75), and IGF-1R expression was positive in 82.67% of the tumor tissues (62/75). Furthermore, 48 samples (64%) exhibited EGFR and IGF-1R co-expression.

Preparation of enediyne-energized fusion proteins. The fusion protein EGF-LDP-IGF, EGF-LDP and LDP-IGF were constructed, extracted and purified according to our previous approach (26). The enediyne-energized analogues of fusion proteins EGF-LDP-IGF-AE, EGF-LDP-AE and LDP-IGF-AE were generated after the active chromophore (AE) of LDM was assembled into fusion proteins. Four enediyne-energized fusion proteins were successfully prepared as measured by reverse-phase HPLC (23).

Binding affinity of the fusion protein EGF-LDP-IGF to ESCC cells. The binding affinity of EGF-LDP-IGF to ESCC cells was analyzed by immunofluorescence staining. KYSE450 cells with high EGFR and IGF-1R expression were incubated with EGF-LDP-IGF protein. Following incubation with anti-His-tag antibody and Alexa Flour 488-labeled antibody, the cells were observed under a confocal laser scanning microscope. As shown in Fig. 2, there was green florescence

located on the membrane and cytoplasm of the KYSE450 cells which indicated that the EGF-LDP-IGF protein was able to bind with the receptors on the cell membrane, and then internalized into the cytoplasm through receptor-mediated endocytosis.

Cytotoxicity of enediyne-energized fusion proteins in vitro. The cytotoxicity of the bispecific fusion protein EGF-LDP-IGF-AE on four ESCC cell lines was assessed by MTT assays. The naked LDM and corresponding mono-specific proteins (EGF-LDP-AE and LDP-IGF-AE) were also tested for comparison. The bispecific protein EGF-LDP-IGF-AE exhibited potent cytotoxic effect on the different ESCC cell lines with IC_{50} values between 10^{-10} and 10^{-15} mol/l (Fig. 3A and B). LDM and mono-specific enediyne-energized fusion proteins EGF-LDP-AE and LDP-IGF-AE also showed strong cytotoxic activity against the four ESCC cell lines. The IC_{50} values analyzed by one-way ANOVA and Dunnett's multiple comparison tests revealed that there were significant differences between EGF-LDP-IGF-AE and LDM in the KYSE450 ($P<0.001$) and EC9706 cells ($P<0.05$). In KYSE510 cells, the differences were significant for EGF-LDP-IGF-AE vs. LDM ($P<0.001$), EGF-LDP-IGF-AE vs. EGF-LDP-AE ($P<0.001$) and EGF-LDP-IGF-AE vs. LDP-IGF-AE ($P<0.01$) (Fig. 3B).

To elucidate whether the phosphorylation and total expression level of EGFR and IGF-1R in the ESCC cells was related to the cytotoxicity of EGF-LDP-IGF-AE, we detected the levels of phospho(p)-EGFR, p-IGF-1R and total EGFR, IGF-1R in the four different ESCC cell lines using western blot assay, followed by densitometry analysis of the band intensity by ImageJ software, and the correlation analysis was carried out using Prism 5 software. The results revealed that there was no significant correlation between the IC_{50}
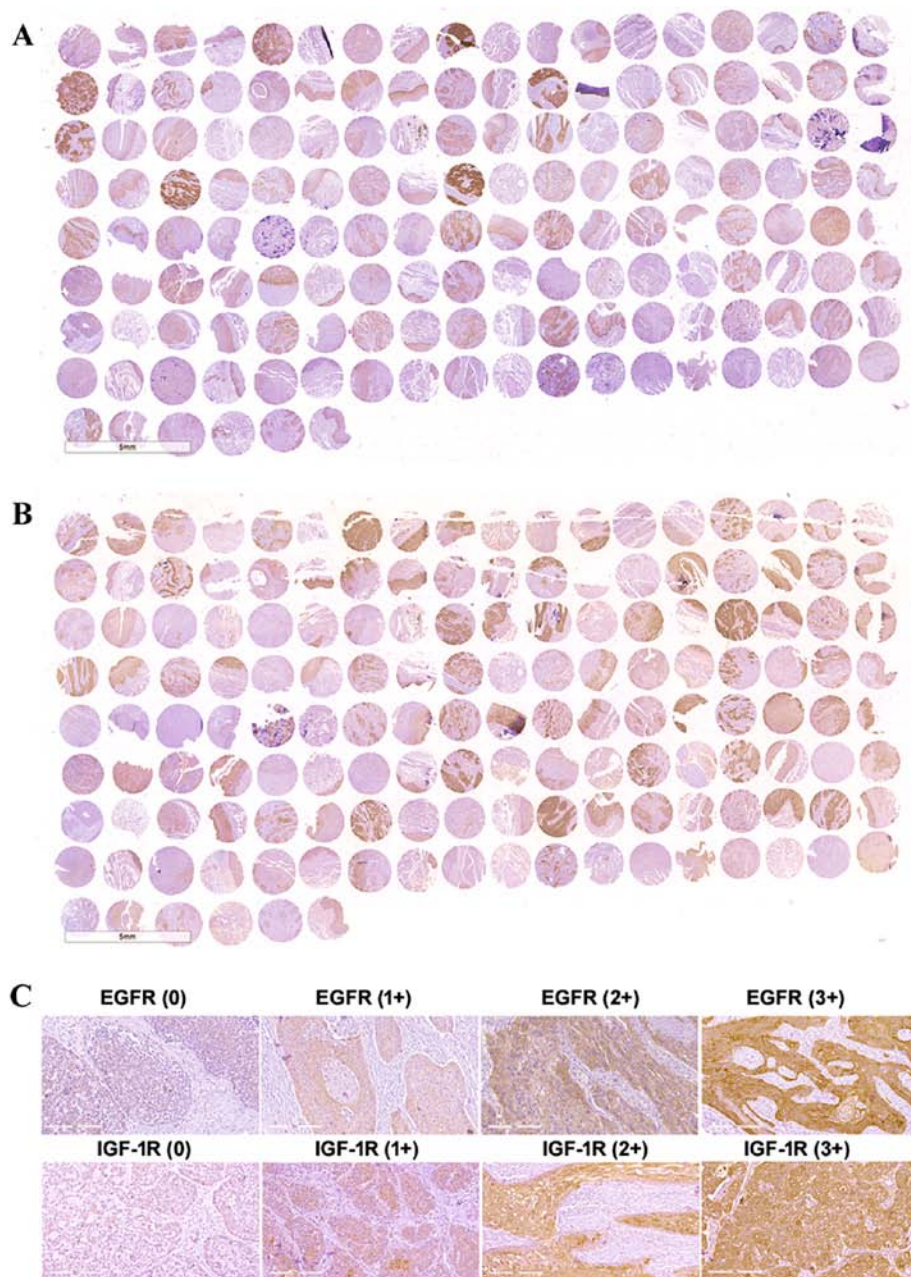
Figure 1. Immunohistochemical analysis of EGFR and IGF-1R expression in ESCC tissue microarrays. Overview of (A) EGFR and (B) IGF-1R expression patterns in ESCC tissue microarrays. Column 1, 3, 5, 7, 9, 11, 13, 15 and 17 include samples of ESCC tissues. Column 2, 4, 6, 8, 10, 12, 14, 16 and 18 include samples of paired adjacent normal tissues. (C) Representative examples of negative and positive staining with intensities of 1+, 2+ and 3+ for EGFR and IGF-1R expression. The images were observed under a microscope at a magnification of x200.
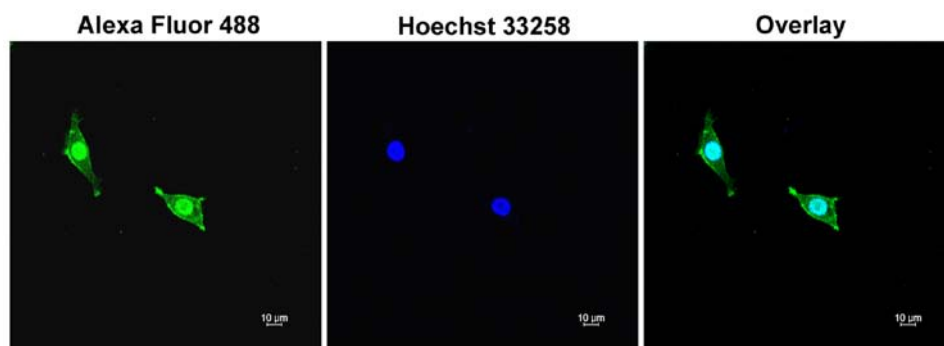

Figure 2. Binding affinity of EGF-LDP-IGF protein to esophageal cancer cells as analyzed by immunofluorescent staining. KYSE450 cells were incubated with EGF-LDP-IGF protein and then exposed to mouse anti-His-tag antibody and Alexa Flour 488-labeled goat anti-mouse antibody. The cell nuclei were stained with Hoechst 33258. Cells were observed under a confocal laser scanning microscope.
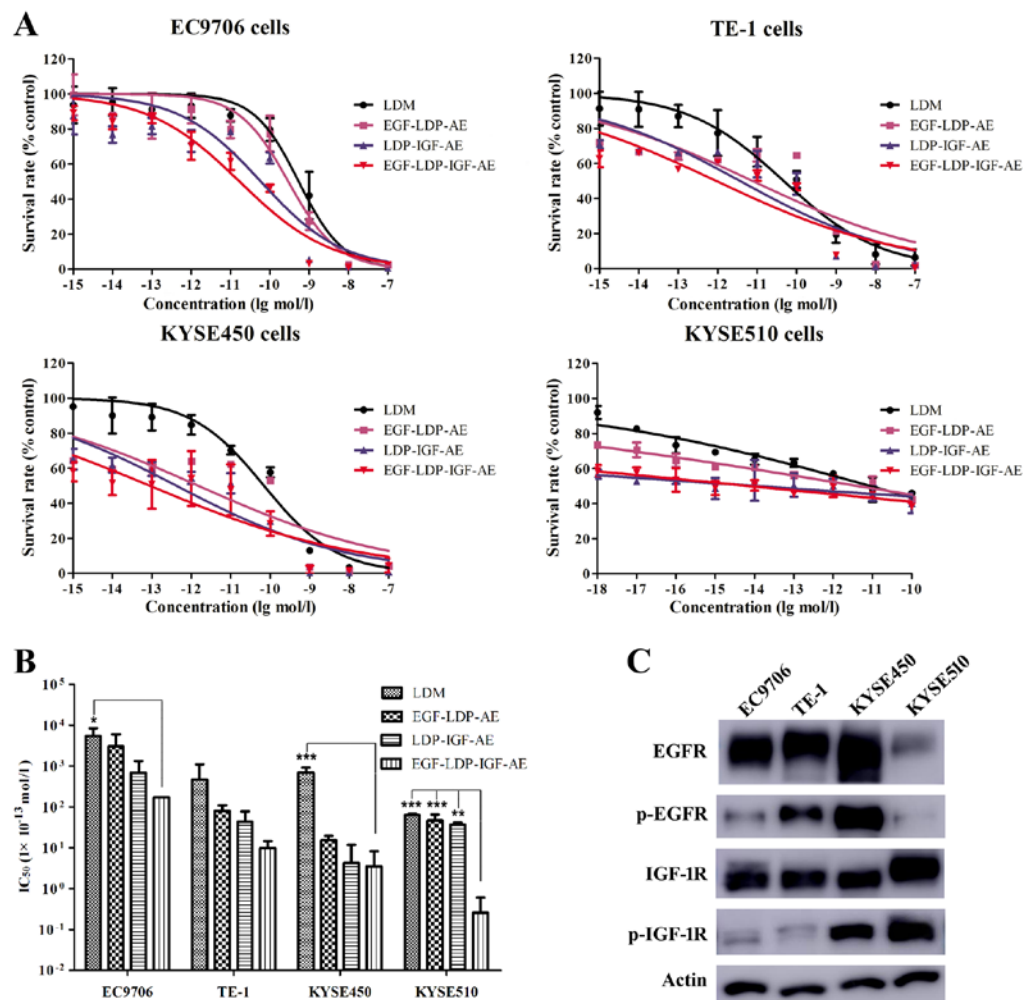
Figure 3. (A) Cytotoxicity of lidamycin (LDM) and enediyne-energized fusion proteins EGF-LDP-IGF-AE, EGF-LDP-AE and LDP-IGF-AE to esophageal cancer cells as determined by MTT assays. (B) The IC_{50} values of LDM, EGF-LDP-IGF-AE, EGF-LDP-AE and LDP-IGF-AE on four ESCC cell lines. All the results were obtained from three independent experiments, and the data are presented as means ± SD; *$P<0.05$ vs. EGF-LDP-IGF-AE, **$P<0.01$ vs. EGF-LDP-IGF-AE, ***$P<0.001$ vs. EGF-LDP-IGF-AE. (C) The phosphorylation and total expression levels of EGFR and IGF-1R in the different ESCC cell lines as analyzed by western blot analysis.

Table II. Phosphorylation and total expression levels of EGFR and IGF-1R in ESCC cell lines and the IC_{50} values for EGF-LDP-IGF-AE against different ESCC cell lines.

Cell line	EGF-LDP-IGF-AE IC_{50} (mol/l) ± SD	Western blot analysis Receptor expression (% actin)			
		EGFR	p-EGFR	IGF-1R	p-IGF-1R
EC9706	$(7.44\pm0.07) \times 10^{-12}$	1.13	0.36	0.83	0.26
TE-1	$(5.83\pm0.02) \times 10^{-13}$	1.08	1.07	1.09	0.22
KYSE450	$(8.47\pm0.98) \times 10^{-14}$	0.91	1.18	1.22	1.17
KYSE510	$(1.19\pm0.04) \times 10^{-15}$	0.56	0.18	1.36	1.20

Quantitative analysis of phospho(p)-EGFR, p-IGF-1R, EGFR and IGF-1R was derived from the results of western blotting in Fig. 3C using ImageJ software. EGFR, epidermal growth factor receptor; IGF-1R, insulin-like growth factor-1 receptor; ESCC, esophageal squamous cell carcinoma.

values of EGF-LDP-IGF-AE and the p-EGFR/IGF-1R or total-EGFR/IGF-1R expression levels (Fig. 3C; Table II).

Effects of bispecific fusion protein EGF-LDP-IGF-AE on cell cycle distribution. After treatment with 0.01, 0.05 and
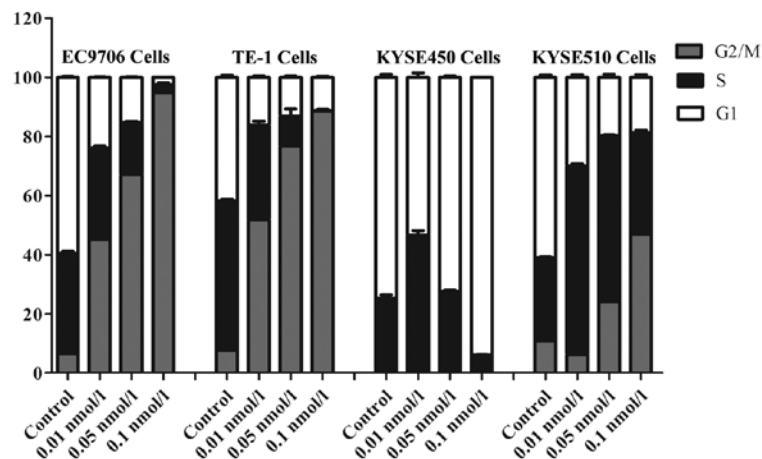
Figure 4. Cell cycle distribution of esophageal cancer cells after treatment with EGF-LDP-IGF-AE at the indicated concentrations for 48 h. Cells distributed in the G1, S and G2/M phases were determined by flow cytometry after PI staining. Results are from three independent experiments and data are presented as means ± SD.
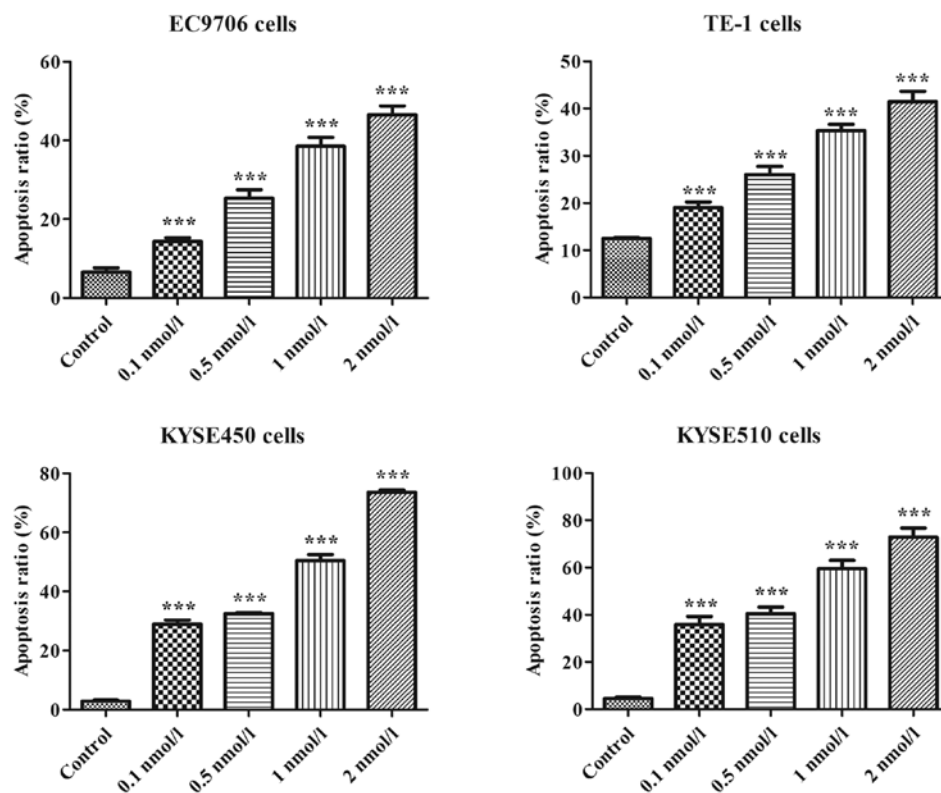

Figure 5. Effects of EGF-LDP-IGF-AE on cell apoptosis. Four ESCC cell lines were exposed to EGF-LDP-IGF-AE for 48 h at the indicated concentrations and the apoptotic cells were detected by flow cytometry after being stained with Annexin V-FITC and PI. Apoptosis ratios are the sum of early apoptotic and late apoptotic cells; ***P<0.01 (EGF-LDP-IGF-AE vs. control).

0.1 nmol/l of EGF-LDP-IGF-AE for 48 h, the ESCC cell lines were stained using PI and the fluorescence was assessed by a flow cytometer. The changes in cell cycle distribution are shown in Fig. 4. The percentages of control cells (EC9706, TE-1 and KYSE510) distributed in the G2/M phase were 6.48±0.78, 7.70±0.16 and 10.87±0.68%, respectively, whereas the percentages of the cells exposed to 0.1 nmol/l of EGF-LDP-IGF-AE which distributed in the G2/M phase were 94.78±0.53, 88.73±0.42 and 46.82±2.28%, respectively. These data

illustrated that a significant G2/M arrest was caused by the EGF-LDP-IGF-AE treatment in the three cell lines. However, data for the KYSE450 cells indicated that an obvious G1 arrest resulted from 0.1 nmol/l of EGF-LDP-IGF-AE treatment (93.85%, 0.1 nmol/l EGF-LDP-IGF-AE treatment vs. 74.6%, control).

Effects of bispecific fusion protein EGF-LDP-IGF-AE on cell apoptosis. The results of Annexin V-FITC/PI staining
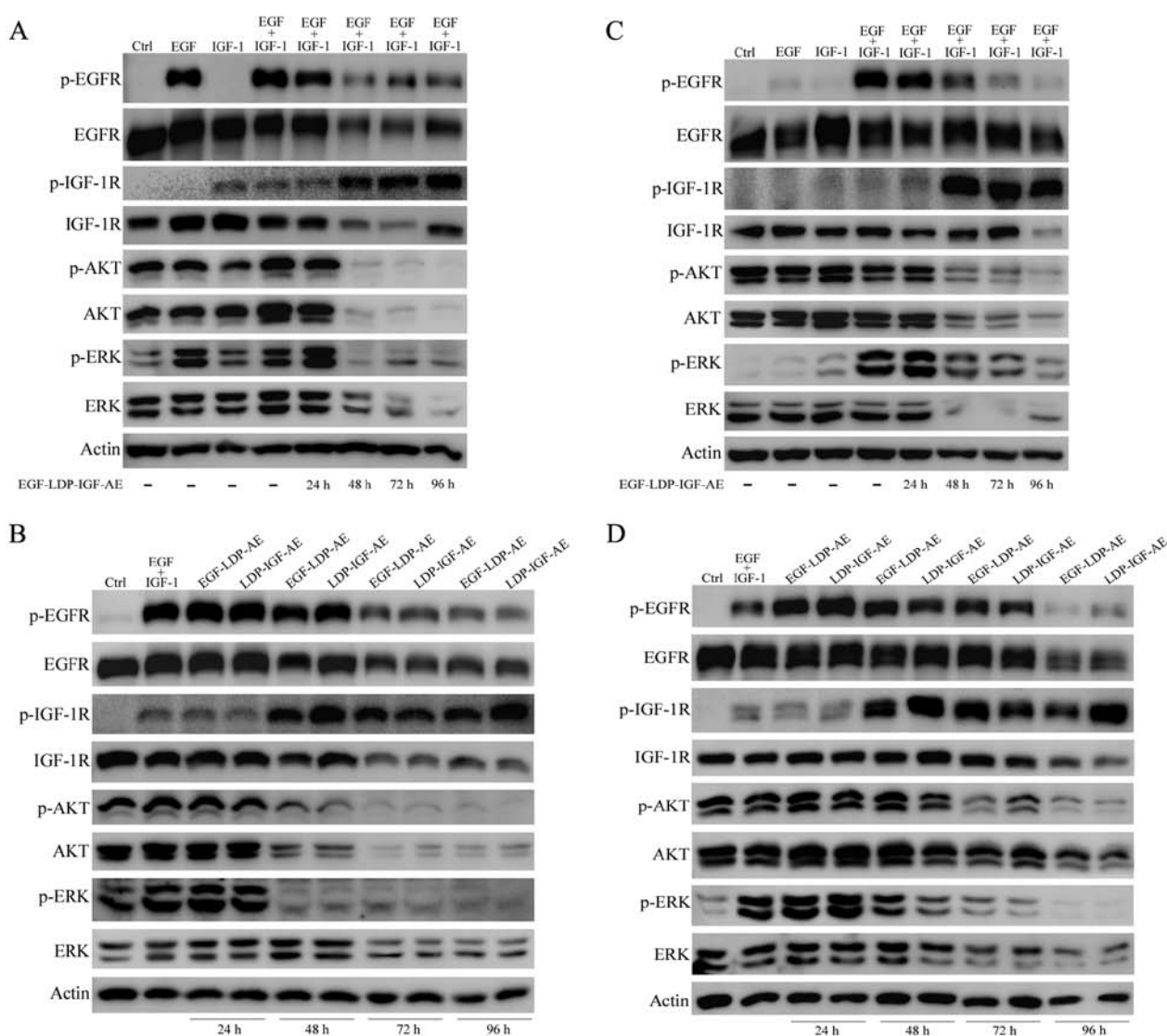
Figure 6. Effects of EGF-LDP-IGF-AE on the EGFR and IGF-1R signaling pathways. (A and C) KYSE450 or EC9706 cells were treated with 0.1 nmol/l EGF-LDP-IGF-AE for 24, 48, 72 and 96 h. (B and C) KYSE450 or EC9706 cells were treated with EGF-LDP-AE or LDP-IGF-AE for 24, 48, 72 and 96 h. Then, the cells were stimulated with human EGF, IGF-1 or both for 30 min. The expression level of key molecules in the EGFR/IGF-1R signaling pathways (e.g. phosphorylated(p)-EGFR, p-IGF-1R, p-ERK and p-AKT) and total EGFR, IGF-1R, ERK and AKT were determined using western blot analysis. β-actin was regarded as a loading control.

assays revealed that the percentages of apoptotic cells (four ESCC cell lines) increased significantly in a concentration-dependent manner after treatment with EGF-LDP-IGF-AE for 48 h. As shown in Fig. 5, the percentages of apoptotic EC9706 cells after treatment with 0.1, 0.5, 1 and 2 nmol/l of EGF-LDP-IGF-AE were 14.32±0.94, 25.35±2.12, 38.53±2.22 and 46.54±2.23%, respectively, which indicated a marked increase compared with that of the control cells (6.56±1.08%; P<0.01). Similar results were also obtained in the other ESCC cell lines (TE-1, KYSE450 and KYSE510) after treatment with EGF-LDP-IGF-AE.

Effects of bispecific fusion protein EGF-LDP-IGF-AE on the activation of EGFR and IGF-1R signaling pathways. EGFR and IGF-1R phosphorylation and downstream signal transduction stimulated by EGF and IGF-1 was regulated by treatment with the EGF-LDP-IGF-AE protein. In addition, the

effect of EGF-LDP-IGF-AE on the EGFR/IGF-1R signaling pathways was closely related to the treatment time. As shown in Fig. 6A and C, in both the KYSE450 and EC9706 cells, the phosphorylation of EGFR and IGF-1R and the two key downstream signaling molecules, AKT and p44/42 MAPK (ERK), as well as their total expression levels were not affected by the EGF-LDP-IGF-AE treatment for 24 h. However, a significant decrease in p-EGFR, p-AKT and p-ERK was observed with the extension of exposure time (48, 72 and 96 h). Interestingly, treatment of EGF-LDP-IGF-AE for 48, 72 and 96 h resulted in a marked increase in p-IGF-1R. The total EGFR and IGF-1R expression levels were decreased after exposure to EGF-LDP-IGF-AE for 48 and 72 h in the KYSE450 cells, but their expression remained unchanged in the EC9706 cells (except for the reduction of total IGF-1R when treated with EGF-LDP-IGF-AE for 96 h). EGF-LDP-IGF-AE treatment for 48, 72 and 96 h also resulted in a
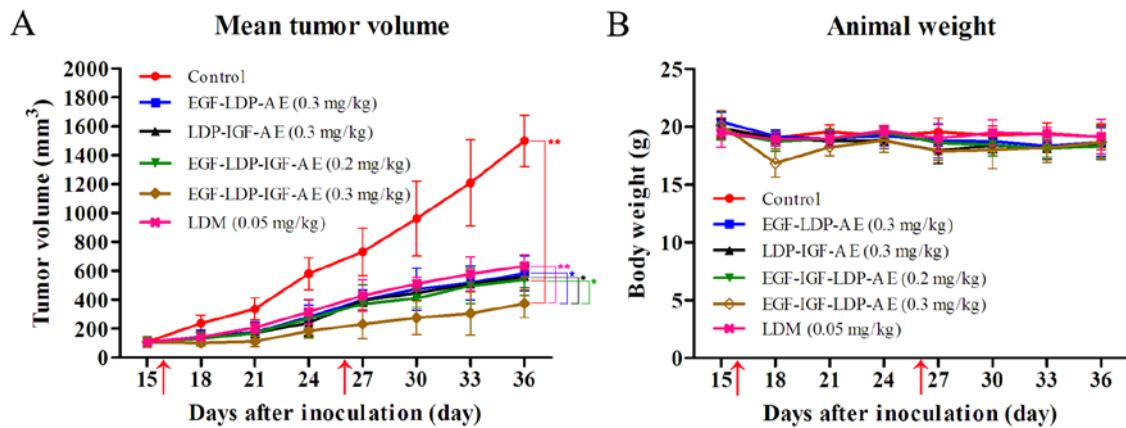
Figure 7. *In vivo* efficacies of LDM and enediyne-energized fusion proteins EGF-LDP-IGF-AE, EGF-LDP-AE and LDP-IGF-AE. (A) The mean tumor volumes and (B) body weights of mice in each group are shown. Red line: **P<0.01, control vs. EGF-LDP-IGF-AE (0.2 and 0.3 mg/kg); pink line: **P<0.01, LDM vs. EGF-LDP-IGF-AE (0.3 mg/kg); blue line: *P<0.05, EGF-LDP-AE (0.3 mg/kg) vs. EGF-LDP-IGF-AE (0.3 mg/kg); black line: *P<0.05, LDP-IGF-AE (0.3 mg/kg) vs. EGF-LDP-IGF-AE (0.3 mg/kg); green line: *P<0.05, EGF-LDP-IGF-AE (0.2 mg/kg) vs. EGF-LDP-IGF-AE (0.3 mg/kg). The arrows indicated the time of injection (day 16 and 26).

marked decrease in total AKT and ERK in both KYSE450 and EC9706 cells (Fig. 6A and C).

The effects of mono-specific fusion proteins EGF-LDP-AE and LDP-IGF-AE on EGFR/IGF-1R signaling were also assessed in the KYSE450 and EC9706 cells. Similar to the bispecific fusion protein, EGF-LDP-AE or LDP-IGF-AE treatment for 24 h did not exhibit effects on the EGFR/IGF-1R signaling pathways. In KYSE450 cells, activation of EGFR was inhibited after exposure to EGF-LDP-AE or LDP-IGF-AE for 72 and 96 h whereas p-IGF-1R was upregulated after treatment for 48, 72 and 96 h. The phosphorylation of two downstream molecules AKT and ERK was significantly reduced after treatment for 48, 72 and 96 h. Total expression level of EGFR, IGF-1R, AKT and ERK was decreased when the exposure time was extended to 72 and 96 h (Fig. 6B). In EC9706 cells, p-EGFR and total EGFR was downregulated only after treatment for 96 h. Levels of p-IGF-1R, p-AKT and p-ERK were significantly altered after treatment for 48, 72 and 96 h, in which p-IGF-1R was increased and p-AKT and -ERK were decreased (Fig. 6D).

Efficacy of enediyne-energized fusion proteins in vivo. In vivo antitumor efficacy of both bispecific and mono-specific enediyne-energized fusion proteins was investigated in a human esophageal cancer KYSE450 xenograft nude mouse model. As shown in Fig. 7A, LDM, EGF-LDP-IGF-AE, EGF-LDP-AE and LDP-IGF-AE significantly suppressed the growth of KYSE450 xenografts. The bispecific fusion protein EGF-LDP-IGF-AE at dosages of 0.2 and 0.3 mg/kg inhibited the growth of xenografts by 64.1 and 75.1%, respectively (P<0.01 compared with the PBS-treated group; P<0.05 between the two EGF-LDP-IGF-AE-treatment groups at different dosages). Furthermore, the EGF-LDP-IGF-AE-treated group at the dosage 0.3 mg/kg showed statistically significant differences (P<0.01) compared with the LDM-treated group at the maximum tolerated dosage (0.05 mg/kg, inhibition rate, 57.8%). Mono-specific fusion proteins EGF-LDP-AE and LDP-IGF-AE at a dosage of 0.3 mg/kg demonstrated similar tumor growth inhibition to bispecific EGF-LDP-IGF-AE

protein at a dosage of 0.2 mg/kg (inhibition rates of 61.2 and 62.6% for EGF-LDP-AE and LDP-IGF-AE respectively). However, when given at the same dosage (0.3 mg/kg), the EGF-LDP-IGF-AE-treated group showed more significant tumor growth inhibition compared with the mono-specific counterparts (P<0.05). No animals died in all groups, and body weight curves showed that the animals tolerated well the administered dosage of the fusion proteins (Fig. 7B).

Discussion

Results from the human ESCC tissue microarray detection in the present study and other previous studies, have revealed that EGFR and IGF-1R are highly co-expressed in ESCC. In addition, the abnormal expression of these receptors is associated with reduced survival, increased risk of relapse and poor prognosis (10,12). Therefore, various EGFR-targeted drugs including monoclonal antibodies (mAbs, cetuximab and panitumumab) and tyrosine kinase inhibitors (TKIs; gefitinib and erlotinib) have been examined in the clinical for esophageal cancer patients. However, the efficacy was far from satisfactory (17-21). Since the crosstalk between EGFR and IGF-1R pathways exist, strategies of the dual-inhibition of both pathways have been pursued for enhanced antitumor efficacy. Strategies include: i) the combination of mAbs or TKIs against different growth factors or receptor tyrosine kinases (RTKs); and ii) bispecific drugs targeting two molecules. Improved antitumor efficacy has been achieved by the combination of mono-specific therapeutic compounds involved in the inhibition of tumor growth, metastasis and anti-angiogenesis (27-30). However, the combination therapy requires the development of individual or combinatorial drugs, which requires a significant investment for production, preclinical and clinical studies. Moreover, some combinations may increase the toxicity and shorten progression-free survival compared to administration of single drugs, such as bevacizumab with panitumumab or cetuximab in advanced colorectal cancer (31,32). Dual targeting strategies with bispecific agents have been classified into two types: i) those that directly act on target molecules,

such as bispecific antibodies and ii) those that depend on targets for delivering an active moiety to killing tumor cells, such as bispecific immunotoxins or fusion proteins. A number of bispecific antibodies targeting both EGFR and IGF-1R (EI-04 and XGFR) have demonstrated superior antitumor activity in preclinical models (33,34), and bispecific immunotoxins/fusion proteins developed by Vallera *et al* also demonstrated either enhanced antitumor activity or broader spectrum of reactivity than the mono-specific molecules (35-37). EGF-LDP-IGF-AE is a bispecific enediyne-energized fusion protein that was constructed by fusing the natural ligands of EGFR and IGF-1R (EGF and IGF-1) to an enediyne antibiotic lidamycin (LDM; C1027) with potent antitumor activity. There are two advantages of EGF-LDP-IGF-AE over the mono-specific fusion proteins. Firstly, the two ligands were designed for receptor binding and subsequent intracellular delivery of the 'warhead', and the LDM acts as toxic moiety for killing tumor cells. The dual-targeting characteristics and the inclusion of the potent cytotoxic payload provide the EGF-LDP-IGF-AE with improved tumor selectivity and enhanced cytotoxicity. Secondly, due to the presence of small targeting ligands (EGF, 6.2 kDa and IGF, 7.6 kDa) and small cytotoxin (LDM, 15 kDa), the EGF-LDP-IGF-AE protein is composed of 253 amino acids with a molecular weight of 27.1 kDa, and the smaller size provides it with enhanced solid tumor penetration, increased tumor uptake and lower immunogenicity. As a result, the bispecific fusion protein EGF-LDP-IGF-AE exhibited potent antitumor efficacy against esophageal cancer.

Binding with EGFR and IGF-1R and internalization were the prerequisites for EGF-LDP-IGF-AE to exhibit its tumor cell-selective cytotoxicity. The results from the immunofluorescent staining assay showed that green fluorescence was located in the membrane and cytoplasm of the ESCC cells, which indicated that the EGF-LDP-IGF protein could bind with the receptors on the cell membrane and then internalize into the cytoplasm through receptor-mediated endocytosis. The bispecific fusion protein EGF-LDP-IGF-AE showed extremely potent cytotoxicity to ESCC cells *in vitro*. However, the correlation analysis revealed that there was no significant correlation between the IC_{50} values of EGF-LDP-IGF-AE and the p-EGFR, p-IGF-1R and total EGFR and IGF-1R expression levels. Similar results were also reported by other studies concerning targeted drugs, such as erlotinib and lapatinib (38,39). We speculated that the mechanisms underlying the internalization of EGF-LDP-IGF-AE into the tumor cells was mainly dependent on receptor-mediated endocytosis. Yet, the AE molecules may dissociate from the EGF-LDP-IGF protein outside the cells, and then the small naked AE molecules enter the cells in receptor-independent mechanisms. This assumption will be further investigated, and the identification of the key molecules to predict the responsiveness to EGF-LDP-IGF-AE may be another focus of further research. This may allow identification of patients who may benefit from the EGF-LDP-IGF-AE-targeted therapy.

In vitro, two-way ANOVA analysis revealed that bispecific EGF-LDP-IGF-AE had stronger cytotoxicity than mono-specific fusion protein EGF-LDP-AE in four ESCC cell lines, but the differences between EGF-LDP-IGF-AE and another mono-specific fusion protein LDP-IGF-AE in KYSE450 and KYSE510 cells were not significant. Actually, LDP-IGF-AE protein was more cytotoxic than EGF-LDP-AE in all ESCC cell lines (P<0.05). The cytotoxicity of fusion proteins depended on the presence of the active enediyne chromophore (AE); therefore, the reconstitution efficiency of AE to fusion protein EGF-LDP or LDP-IGF was closely related to their cytotoxicity. The protein structure of EGF-LDP may affect its reconstitution efficiency, resulting in the lower cytotoxicity. Results from the *in vivo* experiments also revealed a more significant tumor growth inhibition following the EGF-LDP-IGF-AE treatment. EGF-LDP-IGF-AE at a dosage of 0.3 mg/kg yielded tumor growth inhibition of 75.1%, which showed a statistically significant difference compared with the LDM-treated group (P<0.01) and mono-specific fusion protein-treated groups (P<0.05). Furthermore, no mice died in the EGF-LDP-IGF-AE-treated group and weight loss in the mice at the termination of the experiment did not exceed 10% of the pretreatment weight, which indicated that nude mice tolerated well the EGF-LDP-IGF-AE at a dosage of 0.3 mg/kg. This dosage was six times the maximum tolerated dose of LDM. These results revealed that bispecific EGF-LDP-IGF-AE protein was less toxic to normal tissues than naked LDM *in vivo*, and this may be due to the capacity of binding the two receptors of the bispecific protein. Therefore, it preferably bound to the tumor cells highly expressing both receptors instead of binding to normal cells with low expression of one or both receptors. In addition, bispecific fusion proteins may extend the patient coverage which is economically advantageous, as a portion of patients may have EGFR overexpression whereas IGF-1R overexpression may be present in another portion of patients.

To illuminate the mechanisms underlying the cytotoxic effects of EGF-LDP-IGF-AE on ESCC cells, PI and Annexin V-FITC/PI staining assays were used to determine cell cycle arrest and cell apoptosis, and the effects on EGFR/IGF-1R signaling was analyzed by western blotting. The data from cell cycle analysis indicated that EGF-LDP-IGF-AE caused a significant G2/M arrest in the EC9706, TE-1 and KYSE510 cells and a G1 arrest in the KYSE450 cells following 0.1 nmol/l EGF-LDP-IGF-AE treatment. Additionally, EGF-LDP-IGF-AE also induced significant apoptosis in the ESCC cells in a concentration-dependent manner. After treatment with EGF-LDP-IGF-AE for 48, 72 and 96 h, activation of EGFR and the two key downstream signaling molecules AKT and ERK was inhibited and the signal transduction was blocked. However, the level of p-IGF-1R was significantly increased after exposure to EGF-LDP-IGF-AE for 48, 72 and 96 h. This phenomenon could be explained by the fact that EGF-LDP-IGF-AE treatment activated IGF-1R by phosphorylation. Then the activated IGF-1R internalized into the cytoplasm and was transported to the lysosome for degradation. As a result, the cell surface IGF-1R was greatly reduced which resulted in a significant decrease in the interactions between IGF ligands and IGF-1R. Therefore, the signaling pathways mediated by IGF-1R were inhibited. The total IGF-1R expression was reduced after treatment with EGF-LDP-IGF-AE which confirmed our speculation.

In summary, bispecific fusion protein EGF-LDP-IGF-AE demonstrated potent cytotoxicity to ESCC cells and caused

significant cell cycle arrest and apoptosis *in vitro*. It also showed high efficacy in suppressing the growth of human esophageal cancer xenografts *in vivo*. These findings suggest that EGF-LDP-IGF-AE may be a potential candidate for esophageal cancer therapy, which may be developed further for clinical application.

Acknowledgements

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