



Development of a new EGFR antibody antagonist which exhibits potential biological effects against laryngeal cancer

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Background: Laryngeal cancer is a common malignant tumor of the head and neck. Clinical treatment methods mainly include radiotherapy and chemotherapy, but the toxicity and side effects of these treatments seriously affect the quality of life of patients. Currently, there are no specific anti-laryngeal cancer drugs available. Therefore, it is necessary to develop new targeted drugs for laryngeal cancer.

Methods: We established a cell model of laryngeal cancer *in vitro* and a TU686 xenograft model *in vivo*. We then carried out the related research through a series of experiments [including laser confocal microscopy, enzyme linked immune sorbent assay (ELISA) and Western blot].

Results: The results showed that the epidermal growth factor receptor (EGFR) antibody antagonist 6E-C could not only specifically bind to EGFR, but also specifically inhibit the binding of EGF to EGFR. Further analysis indicated that 6E-C could inhibit the EGFR-mediated intracellular signaling pathway. Furthermore, 6E-C inhibited xenograft tumor growth *in vivo*.

Conclusions: In summary, we have successfully prepared a new anti-EGFR antibody antagonist, which exhibited anti-laryngeal cancer effects *in vitro* and *in vivo*. The current research demonstrates that the EGFR antibody antagonist 6E-C shows potential as an effective anti-laryngeal cancer agent, with potential clinical application value. This study therefore provides a solid foundation for related research in the future.

Keywords: Epidermal growth factor receptor (EGFR); signaling; laryngeal cancer; antibody antagonist

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Introduction

Laryngeal cancer is a common malignant tumor of the human head and neck (1,2). The clinical treatment methods mainly include radiotherapy and chemotherapy, but the toxicity and side effects of these treatments seriously affect the quality of life of patients (3,4). Currently, there are no specific anti-laryngeal cancer drugs available. Therefore, scientists have been exploring targeted drugs for laryngeal cancer.

It is well known that epidermal growth factor (EGF) is

closely related to tumor cell proliferation, angiogenesis, tumor invasion, metastasis, and apoptosis (5-7). Many studies have reported that the EGF receptor (EGFR) is overexpressed in head and neck cancers (such as laryngeal carcinoma). EGF interacts with EGFR to carry out its biological roles and functions. EGFR belongs to the tyrosine kinase receptor family. The family has 4 members: EGFR (ErbB1), ErbB2 (Her2), ErbB3 (Her3), and ErbB4 (Her4). These receptors can be activated by 7 ligands, such as EGF and transforming growth factor (TGF- α) (8,9). The first step in EGFR activation is the binding of EGF to

EGFR, inducing EGFR to form a dimer, which in turn leads to EGFR autophosphorylation on multiple residues. EGFR activation further activates EGFR-mediated downstream signaling molecules, such as STAT3 and AKT (10,11). Up to now, studies have shown that EGFR is overexpressed in laryngeal cancer tissues and is closely related to the proliferation of laryngeal cancer cells. Therefore, the development of EGFR antagonists may be a potential treatment option for laryngeal cancer. Traditionally, there are 2 strategies for the preparation of EGFR antagonists. One is to develop small-molecule EGFR tyrosine kinase inhibitors (EGFR-TKIs). For example, the first-generation EGFR-TKIs include gefitinib and erlotinib (12,13). The mechanism of action of erlotinib is to inhibit the binding of the kinase domain, thereby blocking the EGFR-mediated signaling pathway and tumor cell proliferation. Another class of drugs is the anti-EGFR antibody drugs. The mechanism of action of this class of drugs is that they bind to the ligand binding site on EGFR, thereby inhibiting the proliferation and growth of tumor cells. However, due to the high mutation rate of EGFR, the sensitivity of different types of tumors to antibody drugs is inconsistent (10-13). Therefore, it is necessary to develop and screen new antibodies against EGFR based on tumor type.

In the current study, we developed and prepared a series of monoclonal antibodies against EGFR. We then performed a series of screening and identification experiments in laryngeal cancer cell lines, and found that 6E-C could inhibit EGFR-mediated signaling transduction both *in vitro* and *in vivo*. The current work demonstrates that the EGFR antibody antagonist 6E-C shows potential as an effective anti-laryngeal cancer agent, which has laid a solid foundation for future research in this field. We present the following article in accordance with the ARRIVE reporting checklist (available at <http://dx.doi.org/10.21037/atm-21-1839>).

Methods

Antibodies and reagents

Anti-EGFR antibody, anti-STAT3 antibody, anti-AKT antibody, and anti-ERK1/2 antibody were purchased from Abcam. Fetal calf serum (FCS) was obtained from Gibco (USA). Phospho-STAT3, phospho-AKT, and phospho-ERK1/2 antibodies were purchased from CST company (USA). Bovine Serum Albumin (BSA) and PVDF membranes were purchased from Beyotime

Biotechnology (Shanghai, China). Cell culture plates were purchased from Corning (New York, USA). DMEM, hypoxanthine-aminopterin-thymidine (HAT), and HT were purchased from Invitrogen (California, USA). The low-fluorescence PVDF membrane was purchased from Bio-Rad Laboratories. Unless otherwise specified, reagents were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA). Animal Experiments were performed under a project license (No.: 20200506) granted by animal ethics committee of First Hospital of Shanxi Medical University, in compliance with national guidelines for the care and use of animals.

Cell culture

TU686 cells were purchased from ATCC (American type culture collection). The cells were maintained in DMEM/F12 (1:1) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Hyclone) at 37 °C in a 5% CO₂ incubator at saturated humidity.

Preparation of the monoclonal antibody to EGFR

EGFR and Freund's complete adjuvant were mixed and emulsified. Then, the mixture (µg/mouse) was intraperitoneally injected into BALB/c mice. A booster injection was administered using the same method and doses at 14-day intervals. The tail blood of immunized mice was sampled to determine antibody titer. When the anti-EGFR titer reached 1:32,000, the spleen cells from mice with the highest titer were prepared. The spleen cells were then fused with mouse Sp2/0 myeloma cells at a ratio of 1:8 using 50% polyethylene glycol (PEG) 4000. The fused cells were cultured in HAT selection medium containing 20% FBS. After 10 days, antibody-producing cells were cultured in HT medium for another 7 days. Then, enzyme-linked immunosorbent assays (ELISA) were conducted to test the cell supernatants. The monoclonal antibody subtypes were identified using the mouse monoclonal antibody isotyping (mAb) ELISA kit according to the manufacturer's instructions.

Indirect ELISA

EGFR protein (2 µg/mL) was added to each well of a 96-well sterile flat-bottom microplate. After incubation at 4 °C overnight, the plate was rinsed 3 times using PBST, and blocked with 5% sheep serum at 37 °C for 1 h. After washing, cell supernatant was added and incubated for

another 2 h in a humidified incubator at 37 °C. After washing 3 times with PBST, HRP-labeled secondary antibody (1:2,000 dilutions) was added and incubated for another 1 h at 37 °C. After washing again, TMB solution was added and incubated at 37 °C for 10 min. Absorbance was read at 450 nm using a microplate reader (Bio-Rad).

In addition, the phosphorylated signal protein was detected using an ELISA kit according to the kit's instructions.

Detection of cell proliferation by MTT

TU686 cells were grown at 37 °C to the logarithmic growth phase. The cells were then washed with PBS 3 times and digested. The cells were then harvested by centrifugation, and 1×10^4 cells were seeded into a 96-well cell culture plate. The different concentrations of anti-EGFR (6E-C) were added. The cells were cultured for 24, 48, and 72 h. After washing cells 3 times, MTT solution was added and incubated for another 4 h. The absorbance value of each well was checked with a microplate reader.

Analysis of apoptosis by flow cytometry

TU686 cells were grown at 37 °C to the logarithmic growth phase. The cells were collected by centrifugation, and 1×10^6 cells were seeded into a cell culture flask and incubated for 24 h. Anti-EGFR (6E-C, 5 µg/mL) or control antibody (6E-C, 5 µg/mL) was added and incubated for 48 h. After washing, the cell medium was discarded, and the cells were rinsed 3 times. Cells were fixed with 70% ethanol at -20 °C for 2 h. After 3 washes, the cells were collected by centrifugation. The cells were resuspended in 500 µL PBS (containing 0.2% RNase A), and incubated at 37 °C for 0.5 h. The cells were then stained using an Annexin V-FITC/PI staining kit (BD Pharmingen, San Diego, CA, USA). The cell samples were detected by flow cytometry (BD FACSCalibur Flow Cytometer).

RT-PCR analysis

The TU686 cells were collected, and total RNA was extracted using the Trizol kit according to the kit's instructions. UV spectrophotometry was used to determine the content and purity of the total RNA. The purified RNA was reverse transcribed into cDNA using oligo-dT-primers. The cDNA was used as a template for PCR amplification.

Bcl-2 mRNA levels were assayed by RT-qPCR using the following primers: fwd: 5'-CACTCAGCATATGGCGCACGCTGGGAGAACGGGGTAC-3'; rvs: 5'-GCCAAGCTCTCGAGCTATCACCGCATGCTGGGGCCGTACAGTTC-3'. β-actin was used as the reference gene.

Western blot

The cells were collected, then the total cell proteins were extracted using lysis. The BCA kit was used to determine protein concentrations. The protein samples were separated by SDS-PAGE and transferred onto low-fluorescence PVDF (polyvinylidene fluoride) membranes. Membranes were washed 3 times, followed by blocking with 5% BSA at 37 °C for 2 h. After washing, the PVDF membranes were incubated with the indicated primary antibody (1:1,500 dilutions) overnight at 4 °C. The membranes were then washed 3 times in TBS-0.1% Tween-20, and Alexa Fluor 488/555-conjugated secondary antibody (1:1,500 dilutions) was added and incubated for 2 h at 37 °C. After 3 washes with TBST, the PVDF membranes were detected by a fluorescence imaging system (Bio-Rad).

Affinity determination by surface plasmon resonance (SPR)

EGFR was coupled to a GLC chip via 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. The different concentrations of antibodies were then injected. The dissociation constant (K_d) was determined by the ProteOn XPR36 system.

The effect of 6E-C on TU686 migration

TU686 cells were trypsinized and counted. Cells at 2×10^4 /mL were seeded into the upper chamber of the transwell plate. Then, 6E-C was added into the upper chamber, and 600 µL DMEM/F12 with 10% FBS was added into the lower chamber. The TU686 cells were cultured for 24 h. The cells that remained in the upper chamber were removed, and cells on the bottom were stained with crystal violet solution. The cells were then observed under a microscope.

Indirect immunofluorescence assay

TU686 cells were grown to 30–50% confluence on cell slides. For routine immunostaining, the cells were washed and fixed with 4% paraformaldehyde for 15 min at RT,

permeabilized with 0.5% triton for 15 min, and blocked with 3% BSA for 0.5 h. Primary antibodies were added to the cells and incubated overnight at 4 °C. After washing, BSA-diluted secondary antibodies were added and incubated for 120 min at 37 °C. After 3 washes (5 min/wash), the cell nuclei were stained with DAPI. The cell samples were observed and imaged using laser confocal microscopy (Olympus FV3000).

Inhibition of the growth of TU686 xenografts by 6E-C

A xenograft tumor model was established to analyze the *in vivo* effect of 6E-C on laryngeal cancer. A total of 4×10^6 TU686 cells were injected into the flank of BALB/c nude mice. The mice were treated with 6E-C when the tumor grew to a diameter of 3–5 mm (approximately 40–55 mm³). The nude mice were randomized into 3 groups (n=5 per group), and the mice were treated with vehicle, IgG1 (isotype control antibody), or 6E-C. Body weight and tumor size were measured using micrometer calipers. Tumor volume (V) was calculated using the following standard formula: $[(D + d)/2]^3$, where D and d were the larger and smaller diameters, respectively.

Statistical analysis

The data are presented as mean values \pm SD. Data were analyzed for statistical significance by one-way analysis of variance (ANOVA) using SPSS version 25.0. A P value of <0.05 was considered statistically significant.

Results

Identification of the expression of EGFR in TU686 cells

EGFR expression was evaluated by laser scanning confocal microscopy using a commercial EGFR antibody. As illustrated in *Figure 1A*, TU686 cells express abundant EGFR. Furthermore, flow cytometry analysis also indicated that high levels of EGFR were expressed in TU686 cells (*Figure 1B*).

Preparation and identification of the anti-EGFR antibody

After 4 immunizations, the spleen cells from immunized mice were fused with Sp2/0 myeloma cells. In total, we screened 69 monoclonal antibodies against EGFR, among which an anti-EGFR monoclonal antibody was selected

for further identification (termed 6E-C, IgG1 subclass). To further characterize whether 6E-C could interact with EGFR, the following experiments were performed: (I) indirect immunofluorescence showed that 6E-C could bind to TU686 cells (*Figure 2A*); (II) colocalization analysis showed that 6E-C could specifically bind to EGFR (*Figure 2B*); (III) to further demonstrate that 6E-C could specifically bind to EGFR, a competitive receptor binding analysis was performed on TU686 cells by flow cytometry, which indicated that non-fluorescently labeled EGF and 6E-C could inhibit fluorescently labeled-EGF from binding to EGFR in TU686 cells (*Figure 2C*). However, the control antibody had no effect in these experiments. Biacore analysis was performed with immobilized 6E-C, and the results showed an affinity of 2.0×10^{-9} mol/L for EGFR.

Analysis of the effect of 6E-C on TU686 cell proliferation using the MTT assay

The effect of 6E-C on TU686 cell proliferation was evaluated using the MTT assay. As shown in *Figure 3A*, 6E-C (but not the control antibody) could significantly inhibit TU686 cell proliferation.

In addition, we also analyzed the effect of 6E-C on the expression of apoptosis-associated genes (Bcl-2 and Bax), and the results showed that 6E-C down-regulated the expression of Bcl-2 and up-regulated the expression of Bax (*Figure 3B*). Additionally, caspase-3 expression was also up-regulated by 6E-C treatment (*Figure 3C*). We also evaluated the effect of 6E-C on TU686 cell migration, and the results showed that 6E-C inhibited TU686 cell migration (*Figure 3D*).

Inhibition of the EGFR-mediated intracellular signaling pathway by 6E-C

We first tested whether 6E-C itself had agonistic activity by western blot, and results showed that 6E-C had no agonistic activities (*Figure 4A*). Next, we analyzed the antagonistic activity of 6E-C by flow cytometry, which showed that 6E-C significantly inhibited the EGF/EGFR-mediated intracellular signaling pathway in a dose-dependent manner, and 6E-C (5 μ g/mL) almost completely inhibited EGFR-induced intracellular signaling (*Figure 4B*).

The effect of 6E-C on the cell cycle and apoptosis

We analyzed the effect of 6E-C on the cell cycle and

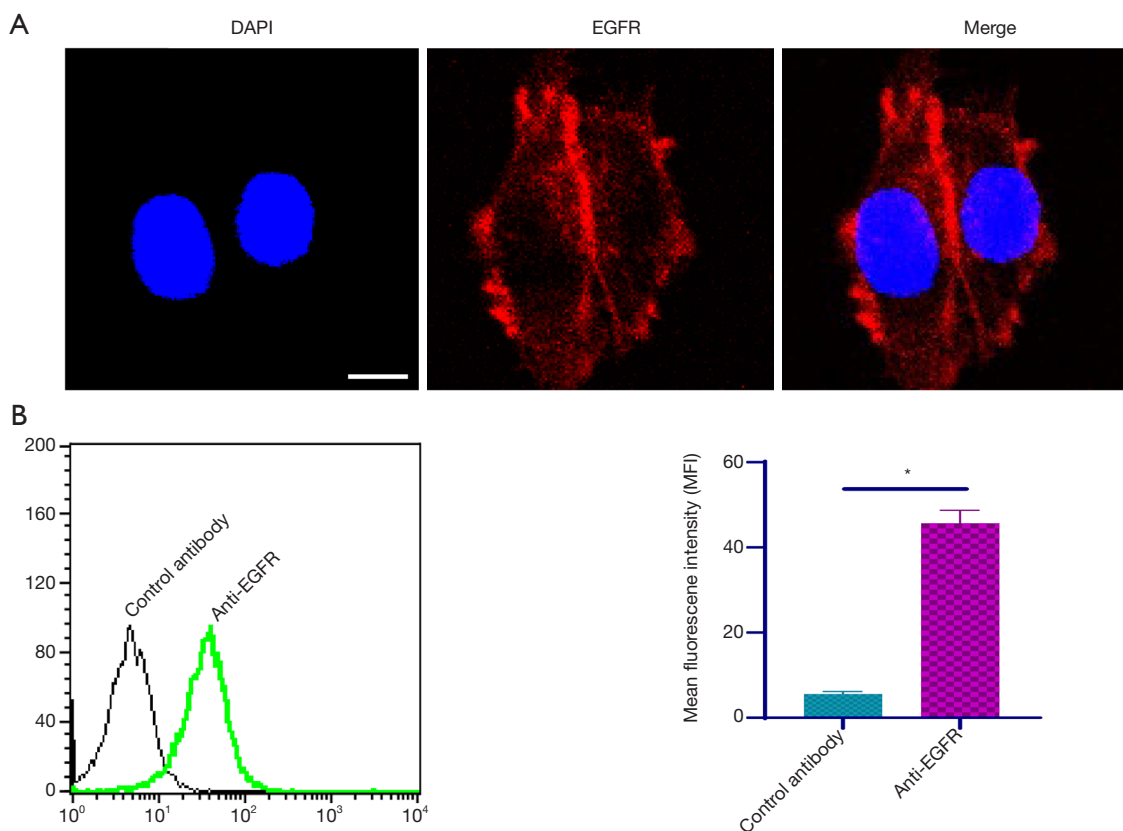


Figure 1 Evaluation of the expression of EGFR. (A) Analysis of EGFR expression by CLSM. (B) TU686 cells express abundant EGFR as determined by flow cytometry analysis. Data are expressed as mean and standard deviation. An asterisk indicates a statistically significant difference ($P < 0.05$). Bar = 10 μm . EGFR, epidermal growth factor receptor; CLSM, confocal laser scanning microscope.

apoptosis of TU686 cells, and the results showed that 6E-C (5–10 $\mu\text{g}/\text{mL}$) altered the cell cycle of TU686 cells, as the proportion of S-phase cells was significantly reduced (Figure 5A). Furthermore, flow cytometry analysis showed that 6E-C significantly increased TU686 cell apoptosis compared to the control antibody (Figure 5B).

6E-C inhibited xenograft tumor growth *in vivo*

A laryngeal cancer xenograft model was established to evaluate the anti-tumor effect of 6E-C *in vivo*. As indicated in Figure 6A, 6E-C (but not the control antibody) inhibited laryngeal cancer xenograft growth (Figure 6). These findings showed that 6E-C has a good anti-tumor effect *in vivo* in nude mice.

The effect of 6E-C on EGFR-mediated intracellular signaling *in vivo*

As shown in Figure 7, 6E-C, but not the control antibody, down-regulated the levels of p-EGFR, P-STAT3, and p-AKT in the xenograft tumor (Figure 7A). We evaluated the cell proliferation of laryngeal cancer tissue by the detection of Ki67, a cell proliferation marker, and results indicated that the percentage of Ki67-positive cells was significantly reduced compared to the control antibody (Figure 7B).

6E-C cellular behavior

The cellular behavior of 6E-C was tested, and results showed that 6E-C was internalized into cells in a time-

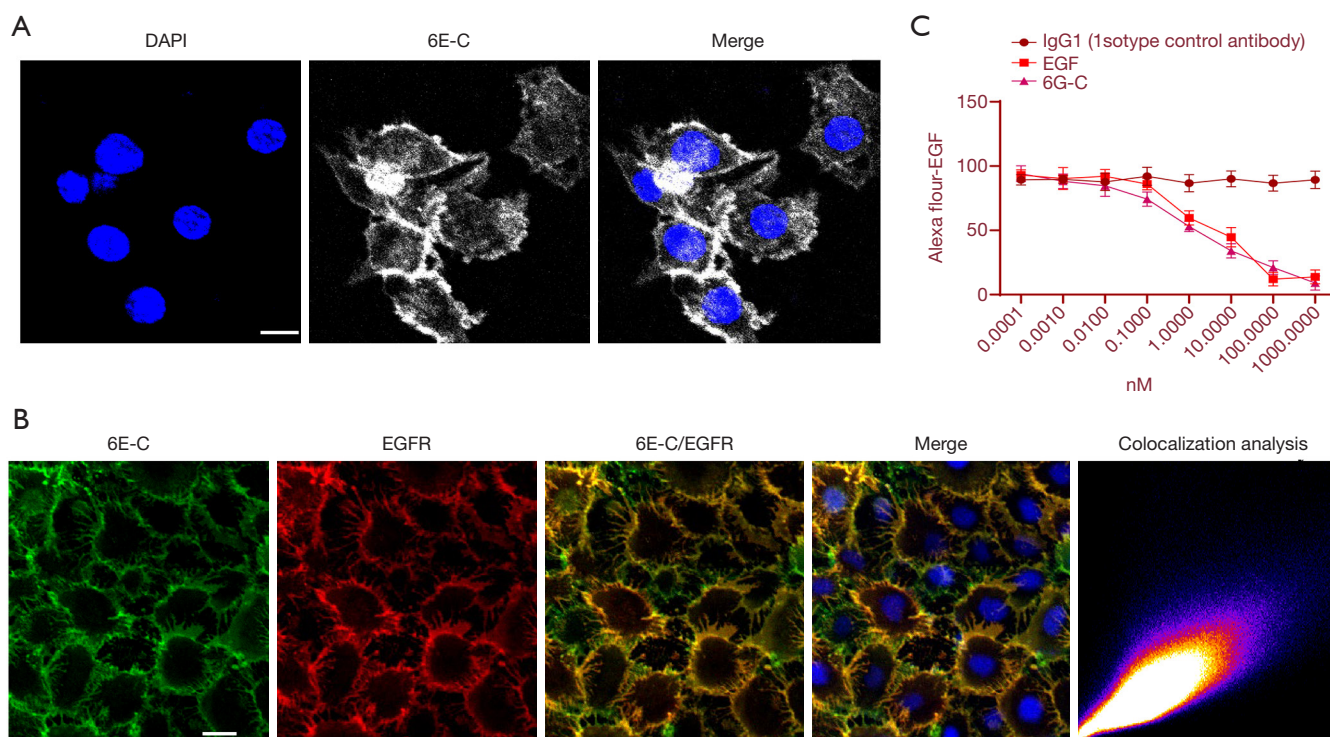


Figure 2 Analysis of interaction between 6E-C and EGFR. (A) 6E-C binds to TU686 cells as shown by the IFA assay. (B) 6E-C binding to EGFR as determined by colocalization analysis. (C) 6E-C could specifically bind to EGFR in TU686 cells as seen through competitive receptor binding analysis. Data are expressed as mean and standard deviation. Bar =10 μ m. EGFR, epidermal growth factor receptor.

dependent manner. At 0–1 min, 6E-C was mainly localized on the cell membrane. Cytoplasmic fluorescence was gradually increased at 30 min, fluorescence intensity reached a maximum value at 60 min, and then the fluorescence intensity began to decline (Figure 8).

Discussion

Laryngeal cancer is a common malignant tumor of the head and neck (14,15). It has been reported that laryngeal cancer occurs mainly in smoking men (16). The clinical treatment methods mainly include radiotherapy and chemotherapy, and no specific anti-laryngeal cancer drugs are available. Therefore, it is necessary to develop targeted drugs for laryngeal cancer. Under normal circumstances, EGF/EGFR has important biological activities, including the regulation of growth and development. From this point of view, EGF/EGFR is a very important biomolecule (17). However, a series of studies have shown that EGFR is closely related to the occurrence and development of laryngeal cancer. For

this reason, in the current study, we developed an antibody inhibitor against laryngeal cancer with EGFR as the target (6E-C). This work showed that 6E-C had good antagonistic effects.

Although researchers have developed many EGFR antibody antagonists, the EGFR mutation rate is high (18-21). Therefore, it is necessary to develop specific EGFR antibody antagonists for different types of tumors. In this study, we prepared and screened a total of 69 monoclonal antibodies against EGFR, among which the anti-EGFR monoclonal antibody 6E-C was selected for further identification. The results showed that 6E-C could not only specifically bind to EGFR, but also specifically inhibited the binding of EGF to EGFR. Further analysis showed that 6E-C could inhibit the EGFR-mediated intracellular signaling pathway.

At present, there are 2 types of EGFR antagonists. One class of molecules are anti-EGFR antibodies, such as cetuximab (21,22). Cetuximab (152-kDa molecule), an anti-EGFR human-mouse chimeric IgG1 monoclonal antibody,

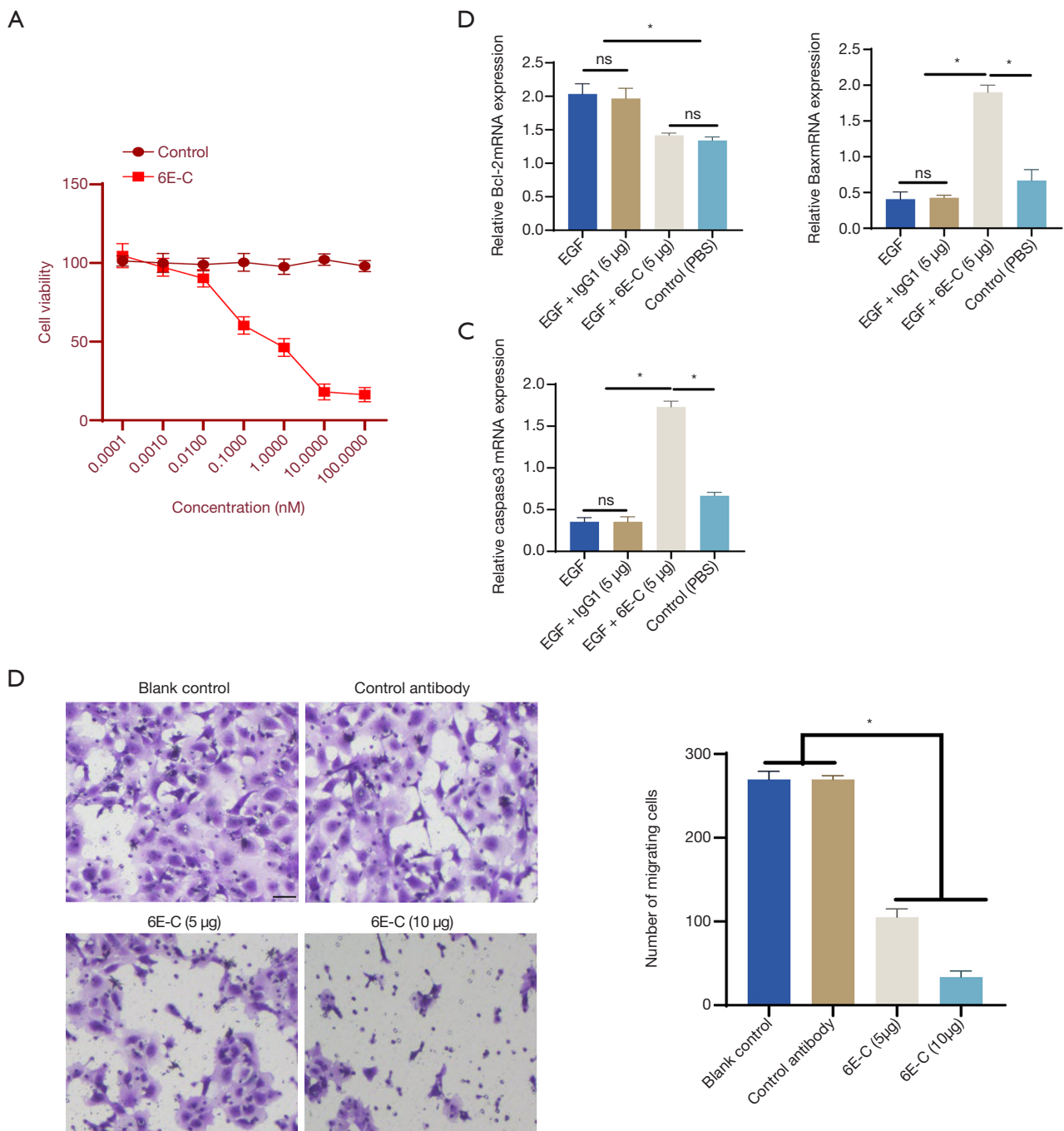


Figure 3 Analysis of the biological activity of 6E-C. (A) The effect of 6E-C on TU686 cell proliferation determined using the MTT assay. (B) The effect of 6E-C on the expression of Bcl-2 and Bax. (C) Caspase-3 expression was also up-regulated by 6E-C treatment. (D) The effect of 6E-C on TU686 cell migration. The non-migrated cells in the upper chamber were removed, the cells in the lower chamber stained with crystal violet. Data are expressed as mean and standard deviation. An asterisk (*) indicates a statistically significant difference ($P < 0.05$). Bar = 50 µm. ns, not statistically significant.

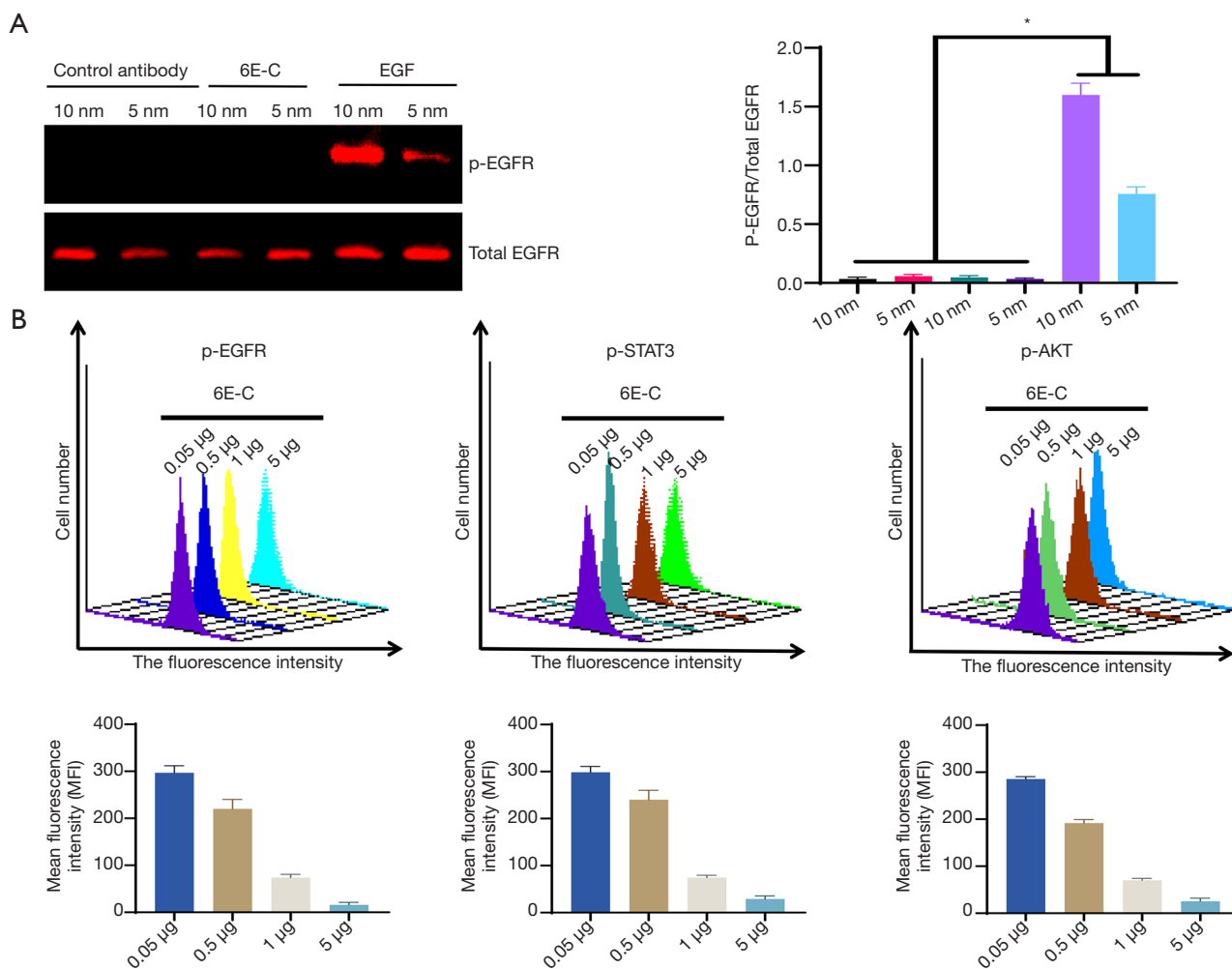


Figure 4 Evaluation of agonistic activity of 6E-C. (A) 6E-C itself has no agonistic activity determined by Western blot analysis. The experiment has been described in detail in the materials and methods section. (B) 6E-C inhibited EGFR-mediated intracellular signaling in a dose-dependent manner. Data are expressed as mean and standard deviation. An asterisk (*) indicates a statistically significant difference ($P < 0.05$). EGFR, epidermal growth factor receptor.

contains the human IgG1 constant region. Cetuximab can inhibit endogenous ligands from binding to EGFR expressed on the cell membrane. Furthermore, it has been reported that cetuximab could promote the internalization and degradation of EGFR. In addition, cetuximab exhibited antibody-dependent cellular cytotoxicity against EGFR-expressing cancer cells. The other types of inhibitors are the EGFR-TKIs. These inhibitors can limit ATP from binding to the intracellular tyrosine kinase domain of EGFR, thereby inhibiting EGFR activation. Subsequently, the EGFR-mediated downstream signaling pathway is blocked. Gefitinib and erlotinib are the first generation of EGFR-TKIs. Recently, a new EGFR antagonist was

developed. Wang *et al.* reported that they developed a new EGFR inhibitor using the anti-idiotypic antibody strategy according to Jerne's idiotypic network theory of the immune system. They prepared an EGFR antagonist (FG8) using hybridoma technology via a series of immunological protocols (23). They found that FG8 could compete with EGF to bind to EGFR. Further research found that FG8 could inhibit the intracellular signaling pathway mediated by EGFR, indicating that FG8 is a potential strategy for the preparation of EGFR antagonists. However, anti-idiotypic antibodies also have their own shortcomings, such as very weak affinity, which is a problem that needs to be solved.

EGFR is closely related to the occurrence and

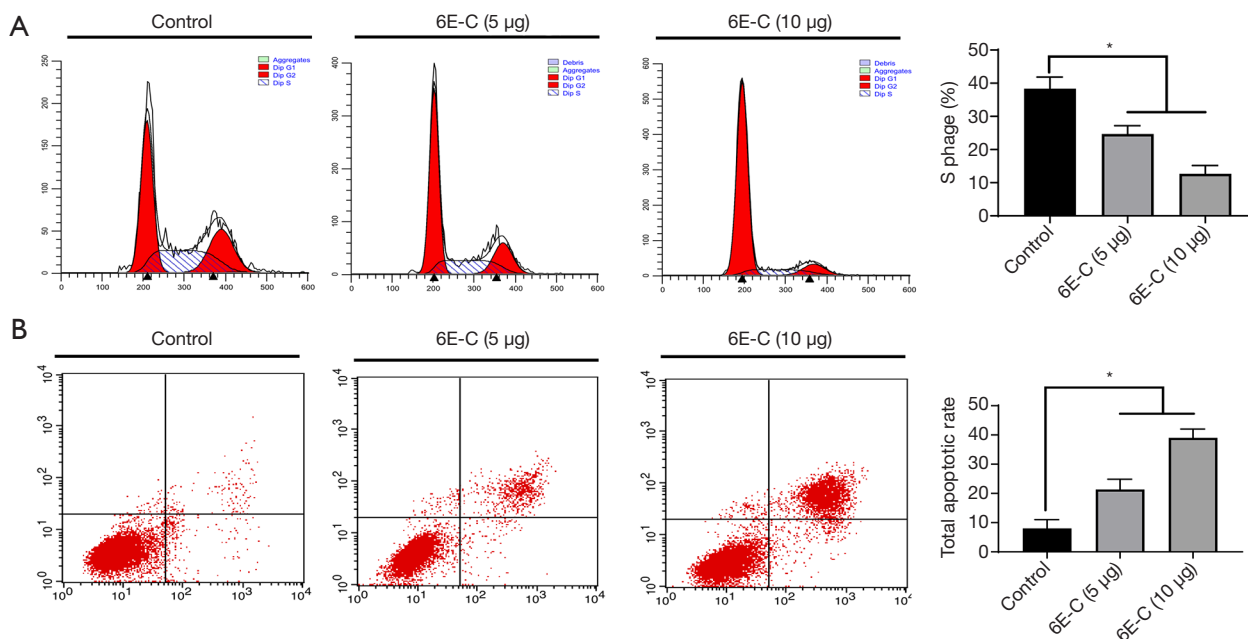


Figure 5 Assessment of the agonistic effect of 6E-C. (A) 6E-C altered the cell cycle of TU686 cells. The experiment has been described in detail in the materials and methods section. (B) 6E-C significantly increased TU686 cell apoptosis. Data are expressed as mean and standard deviation. An asterisk (*) indicates a statistically significant difference ($P < 0.05$).

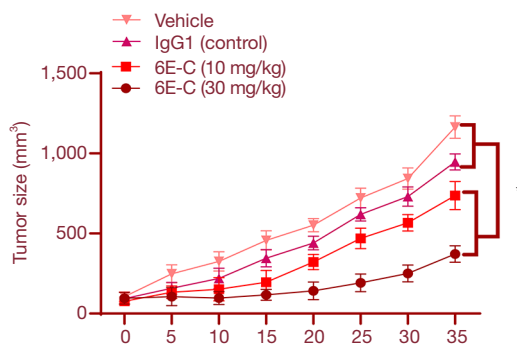


Figure 6 Inhibition of laryngeal cancer xenograft growth by 6E-C. Data are expressed as mean and standard deviation. An asterisk (*) indicates a statistically significant difference ($P < 0.05$).

development of a series of tumors (18). It has been reported that EGFR is significantly up-regulated in many types of tumor cells, such as non-small cell lung cancer, head and neck squamous cell carcinoma, colorectal cancer, breast cancer, and brain cancer (18). Therefore, 6E-C has good application potential in the future, and related experiments are ongoing in our lab.

It is well known that the cellular behavior of EGF/EGFR

is closely related to its biological activities (24). Therefore, we analyzed the cellular properties of 6E-C and found that 6E-C could internalize into cells, suggesting that 6E-C could induce EGFR degradation.

In summary, in the current study, we prepared a monoclonal antibody against EGFR, and found that 6E-C could inhibit EGFR-mediated signaling transduction in *in vitro* experiments. Furthermore, 6E-C has good anti-

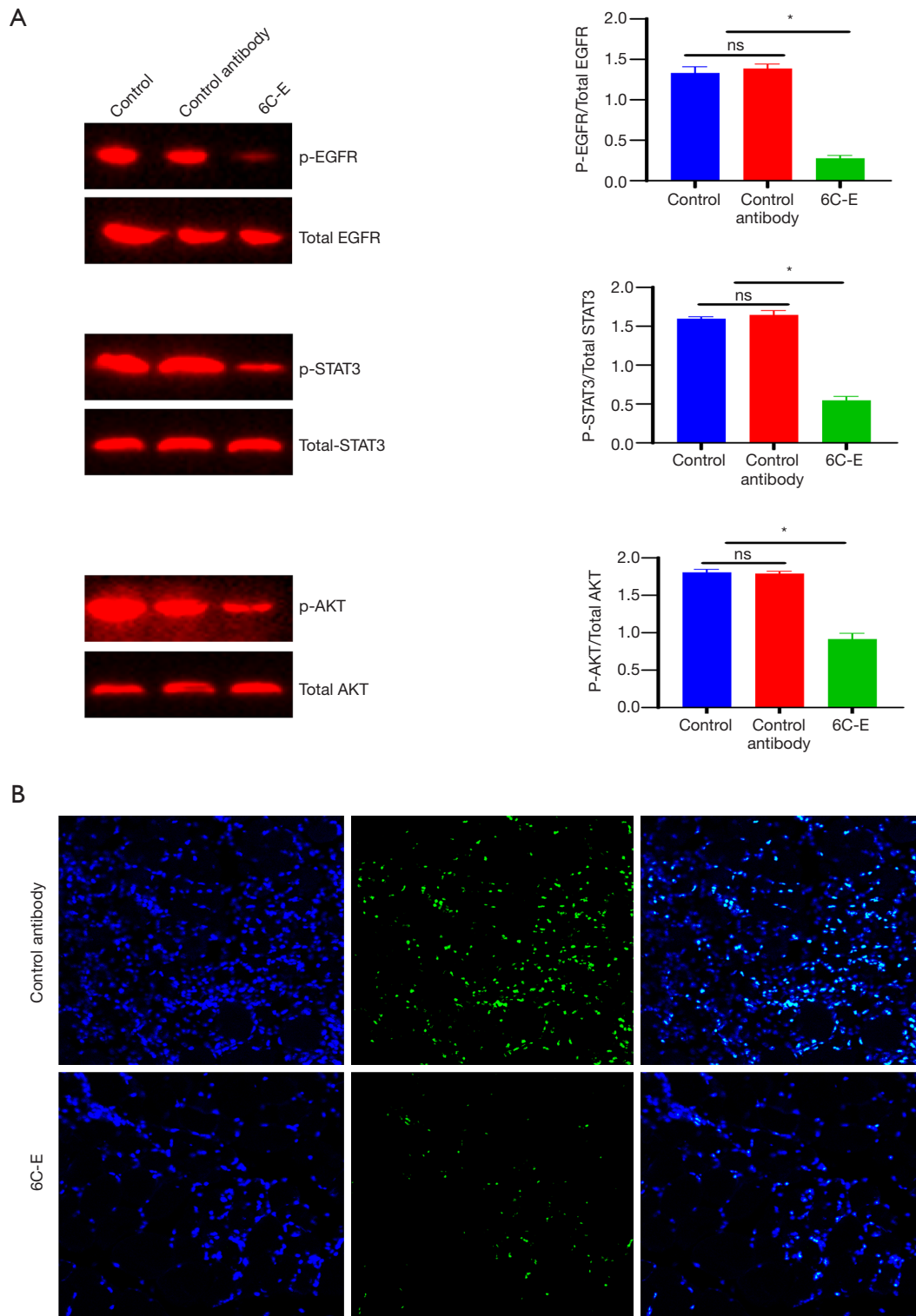


Figure 7 The effect of 6E-C on EGFR. (A) 6E-C down-regulated the levels of p-EGFR, P-STAT3, and p-AKT. (B) The percentage of cells positive for Ki67, a cell proliferation marker, was significantly reduced by 6E-C treatment. Data are expressed as mean and standard deviation. An asterisk indicates a statistically significant difference ($P < 0.05$). Bar = 50 μ m. EGFR, epidermal growth factor receptor; ns, not statistically significant.

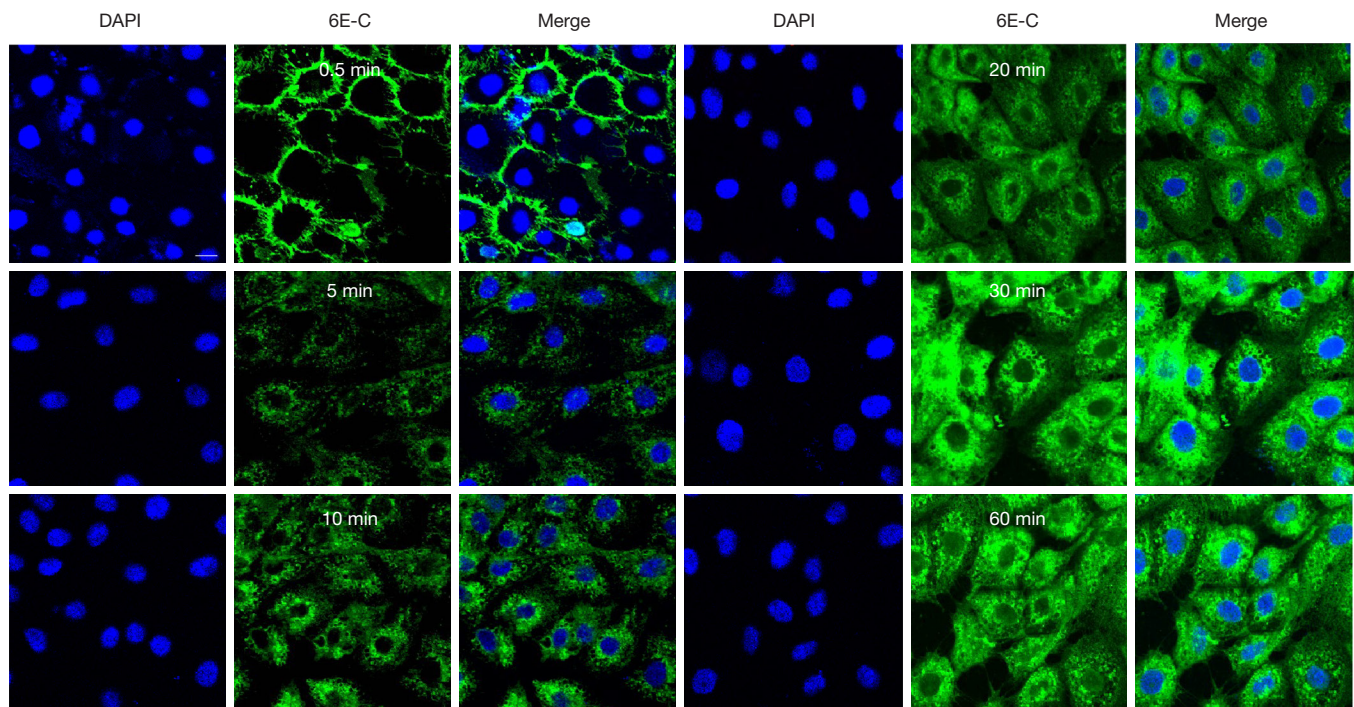


Figure 8 The cellular behaviour of 6E-C. The experiment process has been described in detail in the materials and methods section. Bar =10 μ m.

tumor effects *in vivo* in nude mice. The current research shows that the EGFR antibody antagonist 6E-C has good potential for the treatment of laryngeal cancer.

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Footnote

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Data Sharing Statement: Available at <http://dx.doi.org/10.21037/atm-21-1839>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-21-1839>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal Experiments were performed under a project license (No.: 20200506) granted First Hospital of Shanxi Medical University, in compliance with national guidelines for the care and use of animals.

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