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Time profile of nimotuzumab for enhancing radiosensitivity of the Eca109 cell line

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Abstract. The aim of the present study was to investigate the ability of Nimotuzumab to increase radiosensitivity at different delivery times in the mixed cancer cell line Eca109, to determine the optimal delivery time. Cultured Eca109 cells were classified into five groups: Control with no treatment (O group); irradiation without Nimotuzumab treatment (R group); treatment with Nimotuzumab 24 h prior to or after irradiation (24NR or 24RN group, respectively); and Nimotuzumab combined with irradiation simultaneously (NR group). Following cells reaching the logarithmic-growth phase, cell survival after exposure to Nimotuzumab was evaluated using an MTT assay; thereafter, the 50% inhibitory concentration (IC_{50}) of the cell line was calculated. Cell-survival curves were generated using a colony-forming assay. Flow cytometry analysis was used to detect apoptosis rates and cell-cycle distribution. The expression level of epidermal growth factor receptor was measured in Eca109 cells with western blotting. Growth inhibition was only observed 72 h after exposure to Nimotuzumab. The IC_{50} was 768 μg/ml. At a dose of 0.2 IC_{50} or 0.3 IC_{50}, the sensitization enhancement ratio of radiosensitivity was highest in the 24NR group. Nimotuzumab enhanced radiation-induced apoptosis in Eca109 cells, with the optimal delivery time at 24 h prior to irradiation (P=0.035). The concentration of Nimotuzumab administered was directly proportional to the increase in radiosensitivity of the cells.

Introduction

In China, esophageal cancer (EC) is ranked sixth and second for the number of reported cases and mortality, respectively (1). Additionally, the most common pathological type of EC is esophageal squamous cell carcinoma (ESCC) (2). Of patients with EC, ~70% are diagnosed with advanced and inoperable EC (3). Furthermore, even following treatment with chemotherapy and definitive or neoadjuvant chemoradiotherapy, the outcome remains poor, with a 5-year overall survival rate of <15% (4). It is therefore important to identify and develop novel therapies and treatments for EC.

Recent research has focused on the role of the epidermal growth factor receptor (EGFR) signaling pathways in the progression of EC (5,6). EGFR is a prototypic cell-surface receptor that belongs to the ErbB/HER oncogene family (7). Additionally, EGFR overexpression or mutations have been reported to serve an important role in tumorigenesis in various EC types (8), and also participate in the development of resistance to chemotherapy and radiation (9,10). It has been demonstrated that EGFR inhibitors alone may be used to treat a number of tumor types, such treatments cause fewer side effects compared with traditional chemotherapy (11,12), and notably improve the local control rate when combined with radiotherapy (13,14).

EGFR inhibitors, including small-molecule EGFR tyrosine kinase inhibitors (TKIs) and monoclonal antibodies, are utilized for clinical treatment of EC. TKIs, including gefitinib and erlotinib, repress EGFR phosphorylation and inhibit downstream signals of EGFR (15). Monoclonal antibodies, including cetuximab and nimotuzumab, have the ability to bind to the extracellular domain of EGFR to prevent EGFR receptor dimerization and the activation of its intracellular tyrosine kinase (16). These drugs have been demonstrated to have a notable radiosensitizing effect and are frequently administered in combination with radiotherapy, whether during a short time period or simultaneously (17). However, the optimal delivery time for EGFR inhibitors has not been determined and administration of these inhibitors at the wrong time may reduce the effects of combined therapy (18). In the present study, the aim was to determine the optimal time for administration of nimotuzumab to enhance its radiosensitizing effect in EC.

Materials and methods

Cell culture. Human Eca109 cells were obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and maintained at the Fujian Provincial Key

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Laboratory of Translational Cancer Medicine (Fuzhou, China). Cells were cultured in RPMI-1640 containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified air-incubator with an atmosphere containing 5% CO_2. Notably, the Eca109 cell line has been reported to be contaminated with cervical carcinoma HeLa cells, as reported by Ye *et al* (19).

Small interfering RNAs (siRNAs). siRNAs were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, cells were seeded in a 6-well plate at a density of $5x10^4$ cells/well 24 h prior to transfection. siRNA complexes were added to cells when cultures reached 50% confluence at a final concentration of 50 nM in the absence of serum. Following incubation at 37˚C for 4 h, the culture medium (Opti-MEMI low serum medium; cat. no. 31985-062) was replaced with 2 ml fresh Opti-MEMI medium supplemented with 10% fetal bovine serum (both Thermo Fisher Scientific, Inc.). Cells were cultured under standard conditions (37˚C) for a further 72 h before being examined by western blot analysis.

A total of three different sequences of siRNA used in the experiment, including EGFR $siRNA_1$, EGFR $siRNA_2$ and EGFR $siRNA_3$, which were designed by Invitrogen; Thermo Fisher Scientific, Inc., to determine the most effective RNA interference sequence. For the negative control (NC) a random sequence siRNA(-) was used. NC siRNA(-) forward, 5'-CGU GAUUGCGAGACUCUGAdTdT-3' and reverse, 3'-dTdTGC ACUAACGCUCUGAGACU-5', which were also obtained from Thermo Fisher Scientific, Inc. (Invitrogen; Thermo Fisher Sientific, Inc.). The siRNAs used were as follows: EGFR $siRNA_1$ forward, 5'-UGAUCUGUCACCACAUAAUUACGG G-3' and reverse, 3'-CCCGUAAUUAUGUGGUGACAGAUC A-5'; EGFR $siRNA_2$ forward, 5'-UUAGAUAAGACUGCU AAGGCAUAGG-3' and reverse, 3'-CCUAUGCCUUAGCAG UCUUAUCUAA-5'; and EGFR $siRNA_3$ forward, 5'-UUUAA AUUCACCAAUACCUAUUCCG-3' and reverse, 3'-CGGAA UAGGUAUUGGUGAAUUUAAA-5'.

Western blot analysis. Cells were seeded at a density of $1x10^3$ cells/well in 3-well plates for 48 h and washed for 5 min three times in ice-cold PBS. Protein was extracted using radioimmunoprecipitation assay lysis buffer (Wuhan Boster Biological Technology Co., Ltd., Wuhan, China). Total protein (20 μg/lane) was separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA), followed by incubation with 10 ml 5% skim milk at room temperature for 1 h. A primary antibody against EGFR (cat. no. ab40815; 1:500; Abcam, Cambridge, UK) and β-tubulin (cat. no. 2128; Cell Signaling Technology Inc., Danvers, MA, USA) was used as the loading control at 4˚C overnight. A horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. A0277; 1:2,500; Beyotime Institute of Biotechnology, Shanghai, China) was used as the secondary antibody at room temperature for 2 h. Subsequently, the coloration was completed by DAB (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Images were captured with a Bio-Rad Gel Doc XR and Quantity One v4.6.8 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

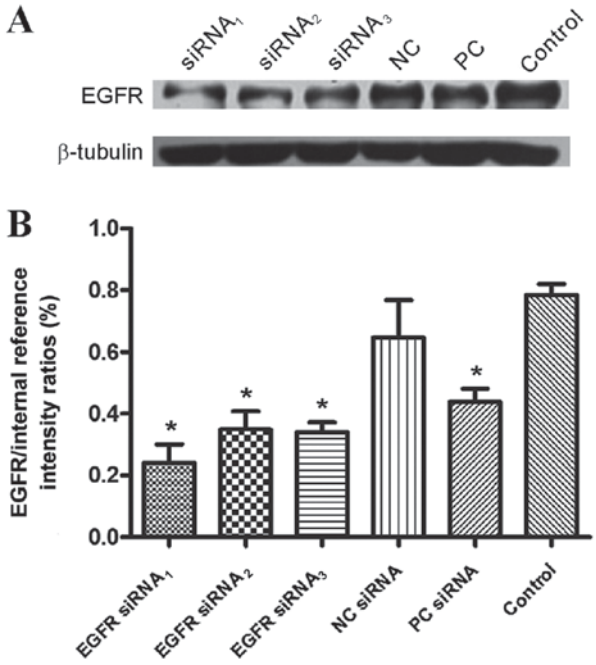

Figure 1. Local expression of EGFR protein determined by western blotting in the Eca109 cell line. (A) Western blot bands. (B) Intensity ratio of target and internal reference bands. A total of 3 different sequences of siRNA (EGFR $siRNA_1$, EGFR $siRNA_2$ and EGFR $siRNA_3$) were used to determine the most effective RNA interference sequence. *P<0.05, compared with group O; siRNA, small interfering RNA; NC, negative control siRNA(-); PC, positive control siRNA(+); EGFR, epidermal growth factor receptor.

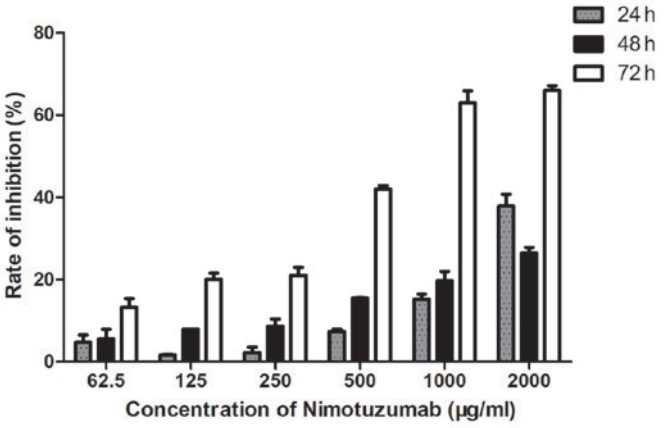

Figure 2. Inhibition effects of nimotuzumab at different concentrations and administered for 24, 48 or 72 h in the Eca109 cell line.

MTT assay. Cells in the logarithmic-growth phase were cultured in 96-well plates at a density of $1x10^5$ cells/well in triplicate. Following incubation for 24 h, nimotuzumab (Trinity Biotech Plc, Beijing, China) was added at concentrations of 2,000, 1,000, 500, 250, 125 or 62.5 μg/ml. MTT (50 μl; Amresco, LLC, Solon, OH, USA) was added following incubation at 37˚C for 24, 48 or 72 h, followed by the addition of 150 μl dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) into each well. A microplate reader was used to determine the absorbance of the formed product at 570 nm [optical density $(OD)_{570}$]. Cell viability (%) was calculated as follows: $(OD_{sample}-OD_{blank})/(OD_{control}-OD_{blank})$ x100. The IC_{50} was also calculated.

Table I. Radiation biology parameters of Eca109 cells fitted using a multi-target model.

Drug doses, μg/ml	Group	D0	Dq	NR	SER
	R	1.65	3.75	2.27	-
150	24NR	1.52	6.64	4.36	1.09
	NR	1.66	2.93	1.76	0.99
	24RN	1.88	2.22	1.18	0.88
200	24NR	1.35	3.24	2.39	1.22
	NR	1.58	2.88	1.81	1.04
	24RN	1.68	3.36	2.0	0.98

R, irradiation without nimotuzumab treatment; 24NR, treatment with nimotuzumab 24 h prior to irradiation; 24RN, treatment with nimotuzumab 24 h after irradiation; NR, nimotuzumab combined with irradiation simultaneously; SER, sensitization enhancement ratio; D0, mean lethal dose; Dq, quasi-threshold dose.

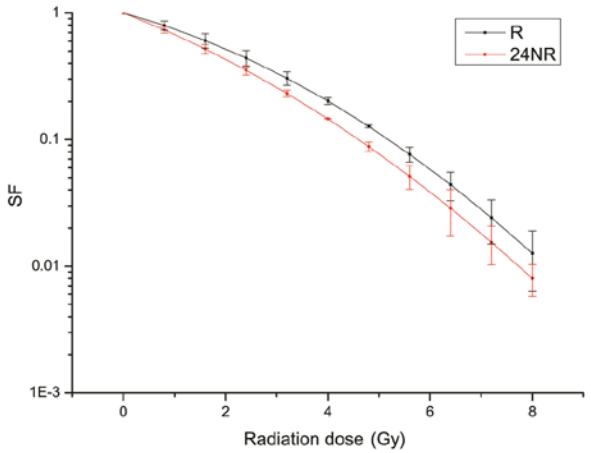

Figure 3. Survival curve of nimotuzumab administration in the 24NR and R groups. SF, surviving fraction; R, irradiation without nimotuzumab treatment; 24NR, treatment with nimotuzumab 24 h prior to irradiation.

Radiation and colony formation assay. Cells were seeded at a density of 6x10^5 cells/well in 3-well plates with a 60-mm diameter. Cultured cells were divided into five groups: Control without any treatment (O group); irradiation without nimotuzumab treatment (R group); treatment with nimotuzumab 24 h prior to irradiation (24NR group); nimotuzumab 24 h after irradiation (24RN group); and nimotuzumab administered with irradiation simultaneously (NR group) Nimotuzumab was administered at different doses, including 2,000, 1,000, 500, 250, 125 and 62.5 μg/ml. Cells were irradiated using a Synergy linear accelerator (Siemens AG, Munich, Germany) at 6 MV exposure with a source-skin distance of 100 cm at a rate of 3 Gy/min and a field of 20x20 cm. Cells were irradiated with doses of 0, 1, 2, 4, 6 or 8 Gy, with three complex holes for each dose. Following irradiation, cells were cultured at 37˚C for 14 days. The number of colonies with >50 cells was recorded by eye. The plating efficiency (%) was determined as follows: (Number of clones/inoculated cells) x100. The surviving fraction (SF; %) was determined as follows: (Colony formation rate of irradiated cells/colony formation rate of control cells) x100. Origin 7.5 software (OriginLab, Northampton, MA, USA) was used to calculate the mean lethal dose (D0), quasi-threshold dose (Dq), SF and sensitization enhancement ratio (SER). The SER of different groups were analyzed at a dosage of 0.2 IC_{50} and 0.3 IC_{50}.

Apoptosis and cell cycle distribution analysis. Trypsin-digested (37˚C for 3 min) Eca109 cells were filtered to prepare a cell suspension. Annexin-V-fluorescein isothiocyanate from Dead Cell Apoptosis kit with Annexin V FITC and PI (eBioscience; Thermo Fisher Scientific, Inc.) was added to the cell suspension for 30 min at 4˚C for labeling. Following washing with PBS for 5 min twice, propidium iodide or 7-aminoactinomycin D staining solution (eBioscience; Thermo Fisher Scientific, Inc.) was added at 4˚C for 30 min, followed by immediate detection of apoptosis in Eca109 cells using a flow cytometer (FACSCalibur™; BD Biosciences, San Jose, CA, USA). The cell cycle was analyzed with the DNA was labeled with nucleic acid dyes.

Statistical analysis. The data were presented mean ± standard error of the mean. One-way analysis of variance followed by a least-significant difference test was performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

EGFR protein expression. A total of three different sequences of siRNA, including EGFR $siRNA_1$, EGFR $siRNA_2$ and EGFR $siRNA_3$, were used to determine the most effective RNA interference sequence. Additionally, NC was included. Subsequently, the gray level of EGFR:NC was 0.7857±0.03581, compared with NC (Fig. 1). The local expression of EGFR was increased in Eca109 cells with siRNAs.

Changes in cell proliferation. Growth inhibition was observed in the cells following exposure to nimotuzumab for 24 and 48 h; additionally, there was growth inhibition following exposure to nimotuzumab for 72 h. The rate of inhibition was increased with the concentration of nimotuzumab, which was notably increased when the concentration was >250 μg/ml. The IC_{50} of nimotuzumab was calculated as 768 μg/ml (Fig. 2).

Colony formation assay. Radiation biology parameters are presented in Table I. The SER of the 24NR, NR and 24RN groups were 1.09, 0.99 and 0.88, respectively, at a dosage of 0.2 IC_{50} (150 μg/ml). The SER of the 24NR, NR and 24RN groups were 1.22, 1.04 and 0.98, respectively, at a dosage of 0.3 IC_{50} (200 μg/ml; Fig. 2). At these concentrations, the 24NR group demonstrated the greatest increase in radio sensitivity, compared with the other groups. Additionally, treatment with increased doses of nimotuzumab proportionally raised the radio sensitivity of cells. The survival curve of the 24NR group at 0.3 IC_{50} demonstrated a notable decrease in SF compared with the R group (Fig. 3). This colony formation assay demonstrated that the 24NR group had reduced D0, compared with the NR group.

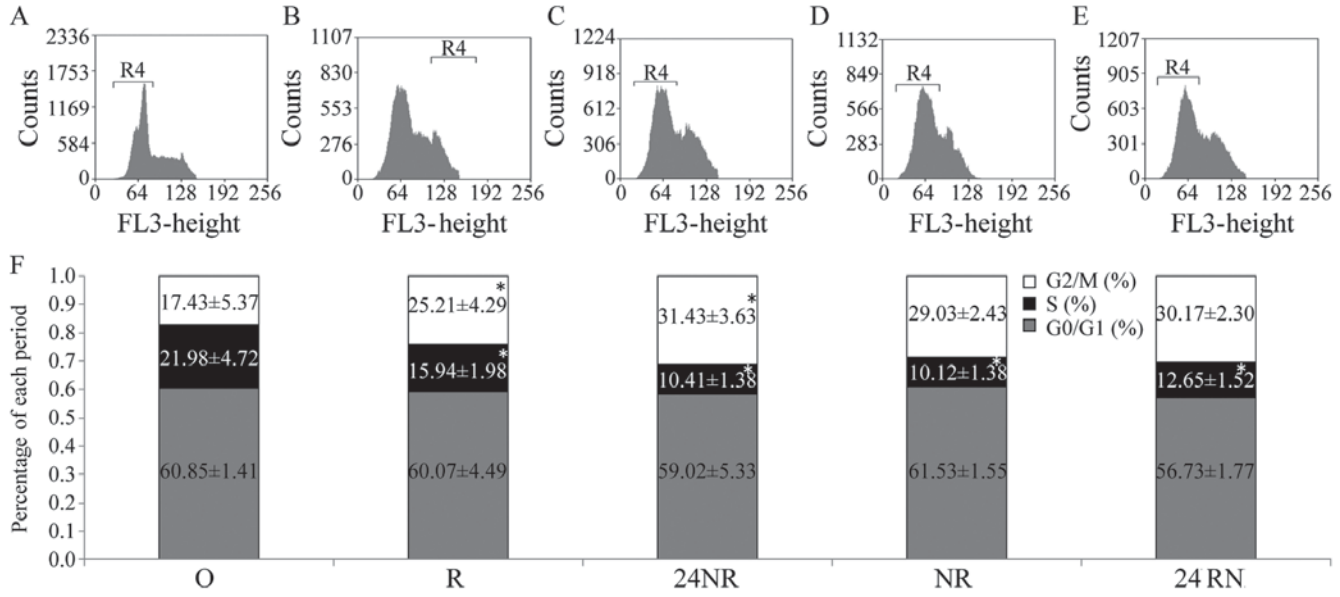

Figure 4. Cell cycle distribution in the five groups. Cell cycle distribution of the (A) O, (B) R, (C) 24NR, (D) NR and (E) 24RN groups. (F) The percentage of each period in the five groups. *P<0.05, compared with group O. O, control with no treatment; R, irradiation without nimotuzumab treatment; 24NR, treatment with nimotuzumab 24 h prior to irradiation; 24RN, treatment with nimotuzumab 24 h after irradiation; NR, nimotuzumab combined with irradiation simultaneously.

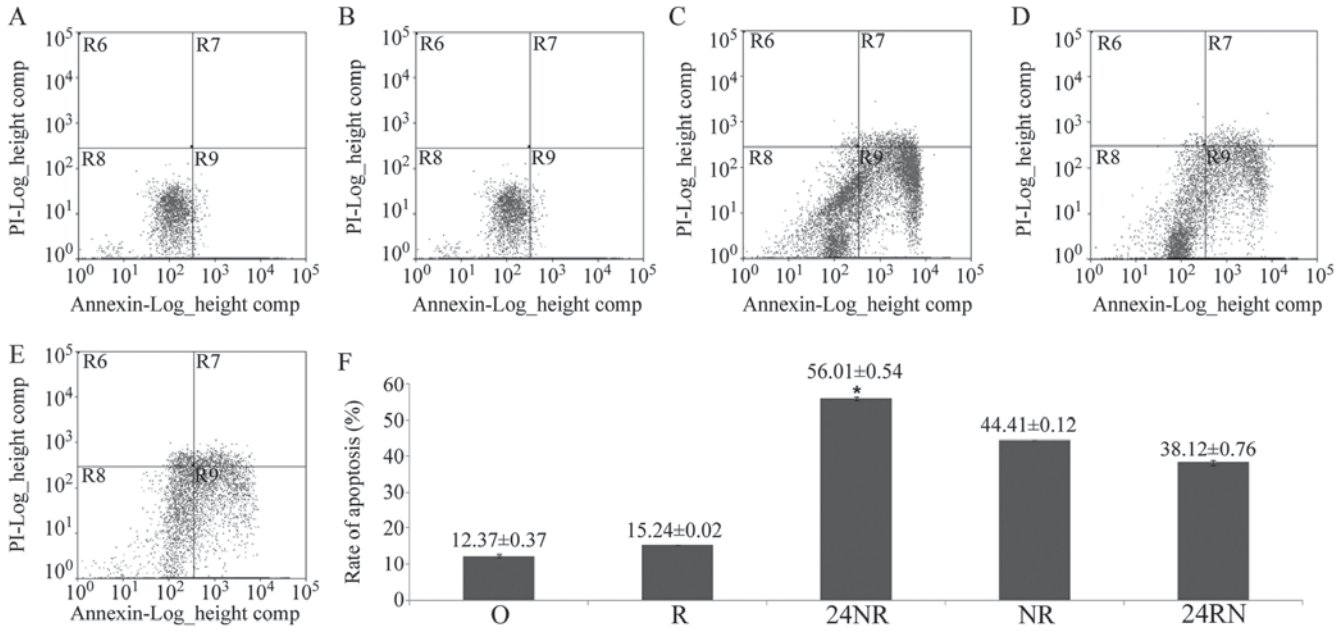

Figure 5. Cell apoptosis rate in the five groups. (A) O group. (B) R group. (C) 24NR group. (D) NR group. (E) 24RN group. (F) The rate of apoptosis in the five groups. *P<0.05, compared with R group. O, control with no treatment; R, irradiation without nimotuzumab treatment; 24NR, treatment with nimotuzumab 24 h prior to irradiation; 24RN, treatment with nimotuzumab 24 h after irradiation; NR, nimotuzumab combined with irradiation simultaneously.

Cell cycle distribution. Cell cycle distribution for each group is depicted in Fig. 4. The percentage of cells in the G_2/M phase in the R group was significantly increased compared with that of the O control group, whereas the percentage of S phase cells was significantly reduced (both P<0.05). In the 24NR, NR and 24RN groups, the proportion of the S-phase cells decreased significantly, while the proportion of G_2/M-phase cells increased compared with the O control group (all P<0.05), indicating that nimotuzumab and radiation exhibit a synergistic effect. Furthermore, the 24NR and NR groups demonstrated significant differences in the proportion of S-phase cells (P=0.041). Although an overall increase in G_2/M-phase cells was evident, no significant differences were reported between the three groups (P=0.62). Additionally, the 24NR group had increased proportions of S and G_2/M-phase cells, compared with the 24RN or NR groups (P=0.53).

Cell apoptosis. Apoptosis rates for each group are depicted in Fig. 5. Nimotuzumab enhanced radiation-induced apoptosis in Eca109 cells at different delivery times. A significantly increased apoptosis ratio was observed in the 24NR (56.01±0.54; P=0.032), NR (44.41±0.12; P=0.025) and 24RN

(38.12±0.76; P=0.036) groups, compared with the R group (15.24±0.02). The apoptosis ratio was significantly increased in the 24NR group compared with NR (P=0.045) and 24RN (P=0.047) groups.

Discussion

In the present study, it was determined that the combination of EGFR inhibition and radiotherapy was more effective compared with individual treatment, which is consistent with another study (20). EGFR is an epidermal receptor with tyrosine kinase activity, which is usually over expressed in epithelial tumor types (21), including epithelial ovarian cancer (22), and breast tumor cells (23). Over expression of EGFR is associated with poor overall survival and increased rates of tumor recurrence, therefore it is an important target for treatment (24). Previous research has demonstrated that EGFR overexpression may regulate cell proliferation and assist tumor cells in avoiding apoptosis (25,26). Additionally, monoclonal antibodies prevent the activation of this intracellular tyrosine kinase, thus inhibiting cell proliferation (27).

Nimotuzumab is a humanized monoclonal antibody. It blocks EGFR, and its downstream signals induce antibody-dependent cell-mediated cytotoxicity, tumor cytotoxicity and effectively stimulate EGFR internalization. Its anticancer properties have been demonstrated *in vitro*, and clinical trials have demonstrated that it is effective for treating tumor types of the head, neck and brain (28-30). Thus far, nimotuzumab has been used to treat >4,000 patients with milder side effects compared with those of cetuximab (31), although cetuximab has also been demonstrated to be more effective against advanced non-small cell lung cancer (NSCLC) (32). Numerous clinical studies have confirmed that treatment with EGFR inhibitors in combination with other therapies is effective and improves the prognosis of patients (33,34). For example, treatment with nimotuzumab in combination with radiation and chemotherapy in head and neck cancer demonstrated positive results (35). Ramos-Suzarte *et al* (34) performed a phase II clinical trial where patients received radiotherapy, alone or combined with nimotuzumab, and determined that the objective response (15.4% vs. 47.8%, respectively) and disease control (26.9% vs. 60.9%, respectively) rates were increased in the combination treatment group, compared with the control group.

The mechanisms of EGFR inhibitors include modifying signal transduction to enhance cellular radiosensitivity, killing cancer stem cells directly, inhibiting repair of DNA damage, reducing repopulation and improving reoxygenation during fractionated radiotherapy (35). Although EGFR inhibitors have been studied extensively, the optimal delivery time for EGFR inhibitors combined with radiation has not been determined, particularly for EC.

In the present study, the effect of delivery time on the effectiveness of EGFR inhibitors was investigated. A colony formation assay demonstrated that the 24NR group reduced D0, compared with the NR group. These results suggest that nimotuzumab enhances the radiosensitivity of Eca109 cells when combined with radiotherapy. The present study also demonstrated that the ratio of S-phase cells in the 24NR group and NR group was significantly reduced, compared with group O (P<0.05), and the proportion of G_2/M-phase cells was increased in the 24NR, NR and 24RN groups, particularly the 24NR group. These results demonstrated that nimotuzumab has a weak effect on cell cycle distribution, but treatment with nimotuzumab 24 h prior to irradiation is most effective. Cells exposed to nimotuzumab in combination with radiation had an increased apoptosis ratio, compared with cells treated with radiation only. Generally, the cell cycle stagnates at the same phase to repair damage and prevent apoptosis (36). Radiation therapy arrested the cell cycle at the G_2/M phase, where multiple growth factors would be required to repair it (37). When the EGFR signal pathway is blocked by nimotuzumab, cells undergo apoptosis as they lack the necessary growth factors (38).

Western blotting demonstrated that EGFR is expressed in Eca109 cells. A number of studies have determined that no significant association between EGFR expression levels and the antitumor effects of EGFR inhibitors (39-41). Garrido *et al* (35) observed that nimotuzumab selectively binds to cells with moderate to high EGFR expression levels, and its antitumor capabilities decreased proportionally with EGFR expression levels. In contrast, the efficacy of cetuximab does not appear to depend on EGFR expression levels. Zhao *et al* (40) demonstrated that nimotuzumab enhances the radiation response and increases the rate of radiation-mediated apoptosis in KYSE30 cells that exhibit high EGFR activity; however, these effects were not observed in TE-1 cells that exhibit low EGFR activity. Akashi *et al* (41) examined the effects of nimotuzumab combined with radiation therapy on human NSCLC cell lines with different EGFR expression levels, and determined that nimotuzumab enhances the effectiveness of radiation therapy in human NSCLC cell lines with high levels of EGFR expression, *in vitro* and *in vivo*. These trials demonstrated that the radiosensitivity of nimotuzumab depends on the expression levels of EGFR. Nimotuzumab and cetuximab are antibodies that inhibit ligand binding upon interaction with EGFR, thereby indirectly inactivating the EGFR kinase. Nimotuzumab has a reduced binding affinity for EGFR, compared with cetuximab. In EGFR-overexpressing cells, nimotuzumab inhibits EGFR-stimulated signaling and ligand-independent basal signaling (42). Additionally, cetuximab is effective at reduced concentrations (43). Compared with the study by Yang *et al* (44), where different cell lines were used, the present study used one cell line. The present study focused on different delivery times in the mixed cancer Eca109 cell line, which is comparative to the study by Yang *et al* (44). Additionally, Yang *et al* (44) focused on the combined use of h-R3 with cisplatin and fluorouracil and determined that the sensitization effect of h-R3 on chemotherapy drugs is associated with the expression level of EGFR in EC1 or EC9706 cells. Furthermore, in the present study it was demonstrated that the concentration of Nimotuzumab administered was directly proportional to the increase in radiosensitivity of the cells (44). The present study demonstrated that treatment with nimotuzumab in combination with radiation affected cell cycle distribution, and enhanced radiation-induced apoptosis and radiosensitivity in human Eca109 cells. The absence of RT-qPCR data is one of the limitations of the present study. Additionally, due to only one cell line being used in the present study, the significance of these data is limited.

In conclusion, the present study demonstrated that the most optimal radiosensitizing effect was observed in the ESCC Eca109 cell line when nimotuzumab was delivered 24 h prior to radiation. The concentration of nimotuzumab administered was directly proportional to the increase in radiosensitivity. Furthermore, due to the effects of nimotuzumab-induced radiosensitization *in vivo* being more complicated than *in vitro*, future studies should focus on *in vivo* experiments to confirm the effects of nimotuzumab.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JL and LW conceived and designed the experiments. LW, YS and JL performed the experiments. ZQ and JL analyzed the data. ZQ and YS contributed reagents, materials and analysis tools.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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