

Nimotuzumab enhances radiation sensitivity of NSCLC H292 cells in vitro by blocking epidermal growth factor receptor nuclear translocation and inhibiting radiation-induced DNA damage repair

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Background: The epidermal growth factor receptor (EGFR) signaling pathway plays a significant role in radiation resistance. There is evidence that EGFR nuclear translocation is associated with DNA double-strand breaks (DSB) repair. Nimotuzumab has shown the effect of radiosensitization in various cancer cells, but little is known about the relationship between nimotuzumab and EGFR nuclear translocation in non-small cell lung cancer (NSCLC) cell lines. In this study, we selected two NSCLC cell lines, namely, H292 (with high EGFR expression) and H1975 (with low EGFR expression) and explored the mechanisms underlying radiation sensitivity.

Methods: MTT assay, clonogenic survival assay, and flow cytometry were performed separately to test cell viability, radiation sensitivity, cell cycle distribution, and apoptosis. Protein γ -H2AX, DNA-PK/p-DNA-PK, and EGFR/p-EGFR expression were further compared both in the cytoplasm and the nucleus with the western blot.

Results: Nimotuzumab reduced the viability of H292 cells and sensitized H292 cells to ionizing radiation. The radiation sensitivity enhancement ratio (SER) was 1.304 and 1.092 for H292 and H1975 cells, respectively. H292 cells after nimotuzumab administration were arrested at the G0/G1 phase in response to radiation. Apoptosis was without statistical significance in both cell lines. γ -H2AX formation in the combination group (nimotuzumab and radiation) increased both in the cytoplasm and the nucleus along with the decreased expression of nuclear EGFR/p-EGFR and p-DNA-PK in H292 cells ($P < 0.05$) that was more significant than that in H1975 cells.

Conclusion: Our research revealed a possible mechanism to explain the radiosensitivity in H292 cells. Nimotuzumab decreased the radiation-induced activation of DNA-PK by blocking EGFR nuclear translocation and impairing DNA DSB repair, thus enhancing radiosensitivity in H292 cells. Because these results represent early research, the matters of how γ -H2AX and DNA-PK dynamically change simultaneously with nuclear EGFR and the best time to administer nimotuzumab will require further exploration.

Keywords: nimotuzumab, NSCLC, EGFR, radiosensitivity, nuclear translocation, DNA-PK

Introduction

Lung cancer is the main cause of cancer death worldwide. Approximately 85% of lung cancer is non-small cell lung cancer (NSCLC). The main treatments for lung cancer include surgery, chemotherapy, radiotherapy, and targeted therapy. Radiotherapy aims to reduce local recurrence and improve the survival of NSCLC patients. Approximately 50% of patients will receive radiotherapy as part of their anticancer regimens. With recent advances in medical technology, radiation dose delivery has become more precise. The incidence of adverse events, such as radiation-induced pulmonary and esophageal

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injury, has decreased,^{1,2} but the prognosis remains dismal with an overall survival of only 16%.³

The progress in radiation techniques should not obscure the fact that radiotherapy is a biological rather than a physical intervention. The “5Rs” of radiobiology are repair, repopulation, redistribution, reoxygenation, and radiosensitivity. Radiosensitization is one of the most promising and thriving research fields aimed at improving radiotherapy effects.⁴

Substantial evidence has shown that many malignant neoplasms highly express epidermal growth factor receptor (EGFR). Approximately 40%–60% NSCLC is with EGFR overexpression.⁵ EGFR overexpression is associated with tumor invasion, metastasis, cycle distribution, apoptosis, and angiogenesis,⁶ and is also responsible for poor prognosis and radiation resistance.^{7,8} EGFR inhibition is a potential method that may enhance radiosensitivity.

Nimotuzumab (h-R3) is a humanized IG1 monoclonal antibody against EGFR. It specifically targets the extracellular epitope and inhibits the combination of ligands, such as EGF. Previous research has shown that the administration of nimotuzumab enhanced the radiation sensitivity of NSCLC cell lines with high EGFR expression.⁹ EGFR inhibitors such as cetuximab demonstrated decreased EGFR nuclear import and suppressed DNA-PK expression in response to radiation, which partially explained cetuximab's radiosensitization mechanism.^{10,11} However, as there has been no such research related to nimotuzumab, its mechanism of radiosensitivity remains poorly understood.

Materials and methods

Cell lines, cell culture, and reagents

The NCI-H292 and NCI-H1975 cell lines (Type Culture Collection of Chinese Academy of Sciences, Shanghai, People's Republic of China) were suspended in RPMI 1640 with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin, and maintained at 37°C humidified atmosphere with 5% carbon dioxide (CO₂). Nimotuzumab was generously provided by Biotech Pharmaceuticals Co, Ltd (Beijing, People's Republic of China) and diluted to certain concentration with RPMI 1640 containing 1% FBS.

Cell arrangement and irradiation

Cells were divided into four groups as follows: control group, nimotuzumab group (h-R3), irradiation group (RT), and the combination group (h-R3+RT). Irradiation was performed 24 hours after nimotuzumab administration using a medical linear accelerator (Primus K; Siemens, Munich, Bayern, Germany) with 6 MV photons, 100 cm focus-surface distance, and dose rate 2 Gy/min. The single X-ray dose ranged from 2–8 Gy.

Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Cells were seeded at 5×10^3 /well in 96-well plates in a final volume of 100 μ L. The original culture solution was discarded 24 hours later, and 1% FBS 1640 solution with or without nimotuzumab was administered to the test well or control well, respectively, for 24 hours, 48 hours, and 72 hours. The nimotuzumab concentration was diluted by half from 1,000 μ g/mL to 500 μ g/mL and dilution continued to 7.8125 μ g/mL. Next, 20 μ L MTT (5 mg/mL) was added to each well and incubated for 4 hours. After the MTT was removed, 150 μ L dimethyl sulfoxide (DMSO) was added to each well. Cell viability was measured by the optical density (OD) of the cell lysates at main wavelength 490 nm and reference wavelength 630 nm (Bio-Rad; Hercules, CA, USA).

Clonogenic survival assay

Cells were planted in six-well plates in triplicate for 24 hours under standard conditions, and then treated with 1% FBS 1640 solution with or without 100 μ L/mL nimotuzumab. Cells received irradiation the next day at distinct doses (0 Gy, 2 Gy, 4 Gy, 6 Gy, and 8 Gy), and the medium was changed to 10% FBS 1640 solution 24 hours later. After 14 days incubation, the colonies were fixed with methanol and stained with 0.5% crystal violet. Colonies containing ≥ 50 cells were counted. The data were fitted into the classic multitarget single-hit model: $SF = 1 - (1 - e^{-D/D_0})^N$ to draw the dose-survival curve with SigmaPlot 10.0 (Systat Software Inc., San Jose, CA, USA). The mean lethal dose (D_0), quasi-threshold dose (D_q), survival fraction at 2 Gy (SF_2), and sensitivity enhancement ratio (SER) ($SER = D_0 \text{ control group} / D_0 \text{ combination group}$) were calculated.

Flow cytometry analysis of cell cycle and apoptosis

Cells were planted in six-well plates and divided into four groups as described above. We then administered 100 μ L/mL nimotuzumab for 24 hours and the cells received 6 Gy irradiation the next day. The cells were harvested 48 hours later and fixed overnight with 70% ethanol. They were then resuspended with PBS containing 100 μ g/mL RNAase and 50 μ g/mL propidium iodide (PI). A flow cytometer (FACScan; BD, San Jose, CA, USA) was used to test the DNA content and cycle distribution. The quantification of cells in different phases was analyzed using CELLQuest (BD, San Jose, CA, USA).

As for the apoptosis assay, 48 hours after irradiation the cells were collected, resuspended with 500 μ L binding buffer, and stained with 5 μ L Annexin-V-EGFP and 5 μ L PI according to the operation manual (Annexin-V-EGFP/PI apoptosis kit;

Beyotime, Nanjing, Jiangsu, People's Republic of China). Cells were then turned to the flow cytometer for the apoptosis test.

Western blot analysis

Cells pretreated with nimotuzumab received irradiation as described above. Cytoplasmic and nuclear extracts were prepared according to the directions for using the nuclear and cytoplasmic extraction kit (Beyotime, Nanjing, Jiangsu, People's Republic of China) 6 hours after irradiation. Protein concentration was determined by the BCA protein assay kit (Servicebio, Wuhan, Hubei, People's Republic of China). Protein lysates were then separated on an 8% or 12% SDS-PAGE gel depending on the protein molecular weight and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Immunoblots were blocked in 5% protease-free bovine serum albumin for 1 hour and probed with γ -H2AX (Abcam, Cambridge, UK), EGFR and p-EGFR (Cell Signaling Technology, Beverly, MA, USA), DNA-PK (Cell Signaling Technology, Beverly, MA, USA), p-DNA-PK (Epitomics, Burlingame, CA, USA), as well as GAPDH and Lamin B1 (Goodhere, Hangzhou, Zhejiang, People's Republic of China) antibodies. The membranes were continually

incubated with appropriate horseradish peroxidase secondary antibodies (Invitrogen, Carlsbad, NM, USA) after washing, and then we detected protein with a chemiluminescence kit (Invitrogen) and visualized the bands on an X-ray film. GAPDH and Lamin B1 were used as an internal control for cytoplasmic and nuclear proteins, respectively, to balance equal loading.

Statistical analysis

Experiments were performed at least in triplicate. Results were expressed as mean \pm SD. Comparisons between the two groups were assessed by the *t*-test with the Statistical Package for Social Sciences, version 17.0 (IBM, Madrid, Spain). The criterion for statistical significance was $P < 0.05$.

Results

Effects of nimotuzumab on the viability of H292 and H1975 cells

The EGFR expression in H292 cells was much higher than that in H1975 cells.⁹ As shown in Figure 1, the viability of H292 decreased with time and dose escalation, whereas H1975 viability remained almost unchanged.

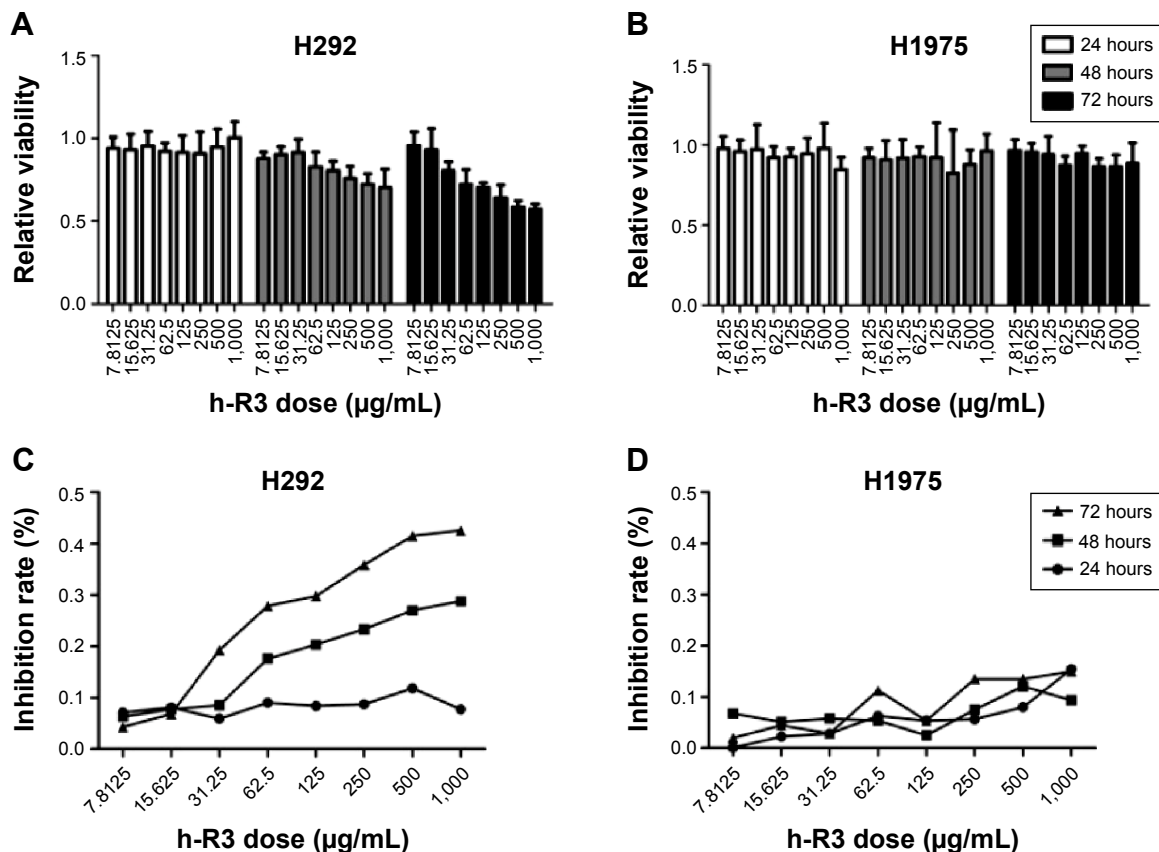


Figure 1 Illustration of nimotuzumab viability influence in H292 and H1975 cells.

Notes: MTT assay revealed that nimotuzumab alone increased H292 viability suppression with time and dose escalation (A and C), but barely influenced H1975 viability (B and D). The suppression ability in H292 cells was still feeble and it was merely 42.5% (calculated separately, not shown in the figure) at maximum dose 1,000 µg/mL.

Abbreviation: MTT, methylthiazolyl-diphenyl-tetrazolium bromide.

The inhibition rate for H292 cells after 72 hours incubation at the highest dose level was less than 50%, suggesting that nimotuzumab alone was not sufficient to suppress cell viability (Figure 1).

Nimotuzumab increased the radiosensitivity of H292 cells

Dose-survival curves indicated that pretreatment with nimotuzumab significantly suppressed the clonogenic survival of H292 cells, but not H1975 cells after varying the dose of

radiation from 2 Gy to 8 Gy (Figure 2; Table 1). The dose enhancement ratio was 1.304 and 1.092, respectively, suggesting that nimotuzumab increased H292 radiosensitivity.

Effects of nimotuzumab in combination with radiation on cell cycle distribution and apoptosis

Flow cytometer assay revealed that a significantly arrested in G0/G1 phase was shown in the combination group compared with the irradiation group in H292 cells ($P < 0.05$),

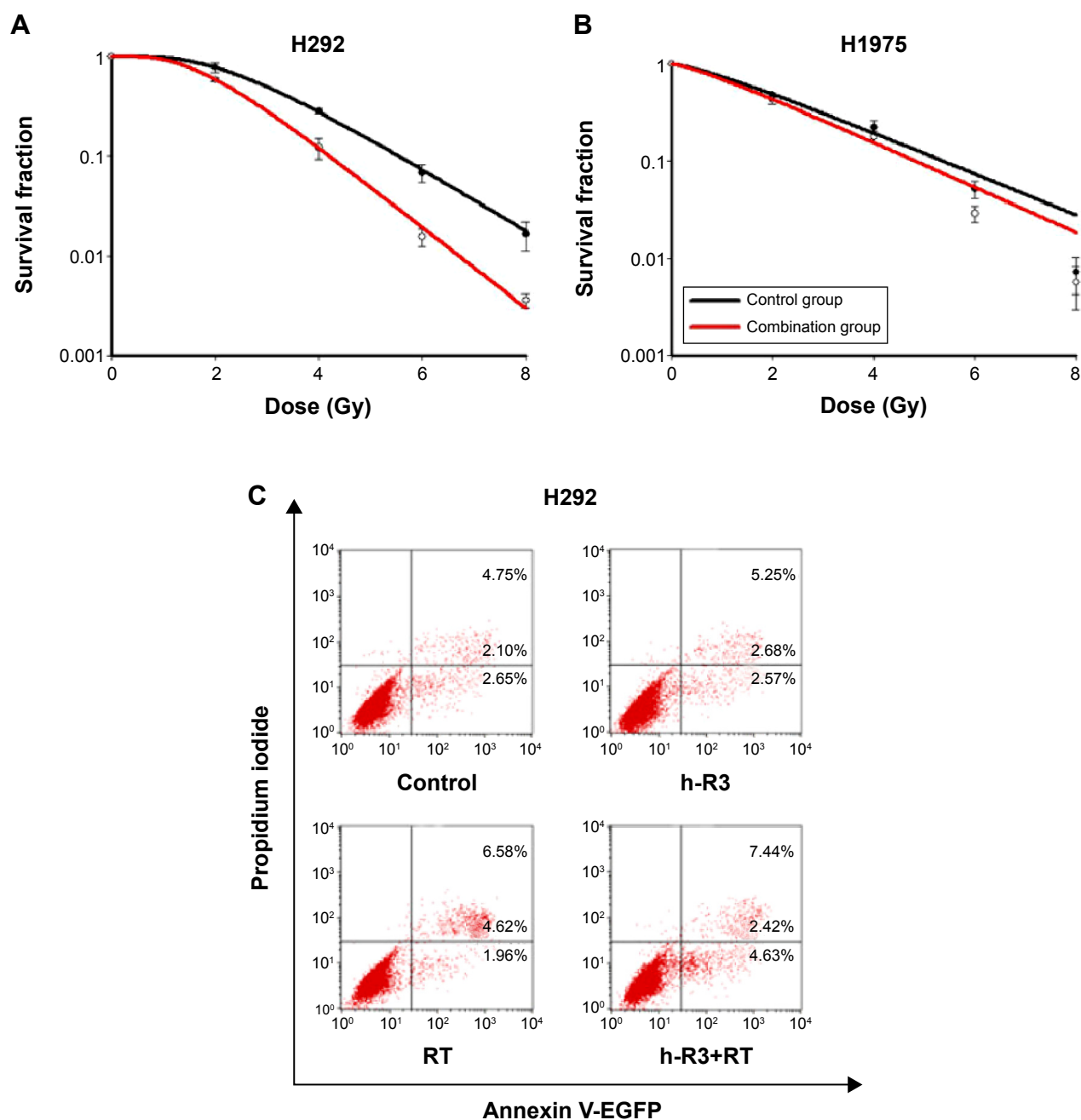


Figure 2 (Continued)

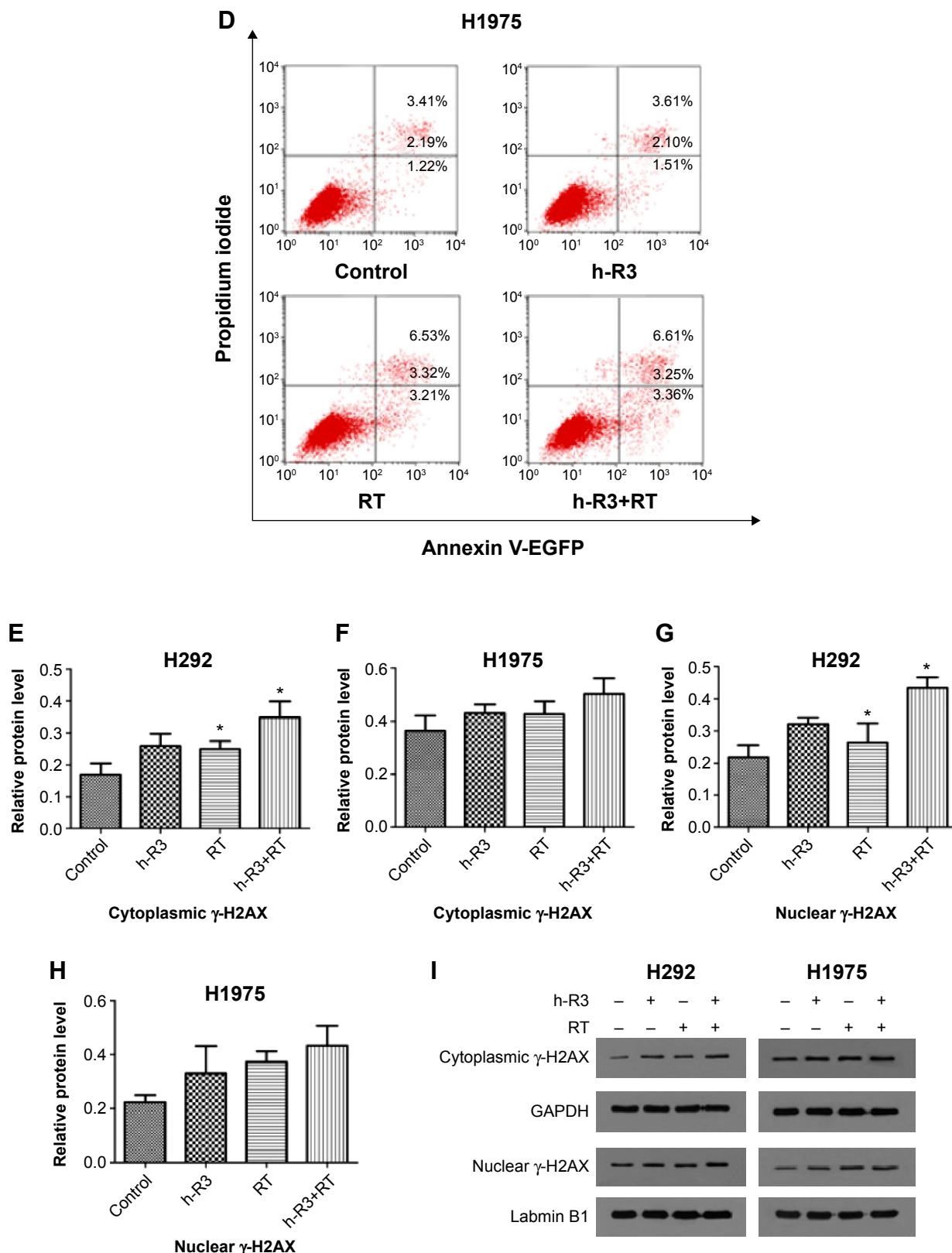


Figure 2 Cell clonogenic survival apoptosis and γ -H2AX formation.

Notes: H292 and H1975 distinct dose-survival curve was illustrated by Figure (A and B) (clonogenic survival assay $n=3$). Cell apoptosis distribution was shown by (C and D). The total apoptosis percentage was marked at the upper space of the right two quarters ($n=3$). There was no statistical significance, but it seemed that H292 cells exhibited the trend of higher apoptotic percentage compared with H1975 cells ($P=0.08$ and $P=0.90$ for H292 and H1975 cells, respectively). γ -H2AX formed in both the cytoplasm and the nucleus. Protein formation was more significant in H292 cells (E and G) compared with H1975 cells (F and H). Figure (I) illustrated the brief profile of protein expression ($n=3$). Each bar represents the mean \pm SD and *indicates significant difference ($P<0.05$).

Abbreviations: h-R3, nimotuzumab group; RT, irradiation group.

Table 1 Radiation parameters fitted to classic multitarget single-hit model

Parameters	H292		H1975	
	Control	Combination	Control	Combination
D0	1.396	1.070	2.039	1.866
Dq	2.053	1.747	1.063	0.922
N	5.581	5.358	1.421	1.347
SF2	0.782	0.592	0.487	0.342
SER	1.304		1.092	

Notes: Control group: cells receiving 6 Gy radiation; combination group: cells pretreated with 100 µg/mL nimotuzumab for 24 hours before receiving 6 Gy radiation; SER = D0 control group/D0 combination group.

Abbreviations: D0, mean lethal dose; Dq, quasi-threshold dose; N, extrapolation number; SF2, surviving fraction at 2 Gy; SER, sensitivity enhancement ratio.

but the difference in H1975 cells was without statistical significance (Tables 2 and 3). The apoptotic cell percentage in the combination group was slightly increased compared to the irradiation group for both cells (Figure 2).

Pretreatment with nimotuzumab increased H292 γ -H2AX formation in response to radiation

To further explore the molecular mechanism of radiosensitivity, we tested the protein expression in both the cytoplasm and the nucleus. The γ -H2AX protein was an important indicator for radiation-induced DNA double-strand breaks (DSB). The γ -H2AX formation was significantly increased in the combination group compared with the irradiation group in H292 cells ($P<0.05$) but not in H1975 cells. Nimotuzumab combined with radiation increased DNA damage in H292 cells (Figure 2).

Pretreatment with nimotuzumab decreased nuclear EGFR/p-EGFR expression in response to radiation in H292 cells

EGFR expression was associated with radiation resistance. Phosphorylated EGFR expression in H292 cytoplasm was more significantly decreased than that in H1975 cells. As for

Table 2 Cell cycle distribution in H292 cells ($\bar{x} \pm s$)

	G0/G1 (%)	G2/M (%)	S phase (%)
Control	77.77 \pm 1.20	9.14 \pm 0.58	13.10 \pm 1.59
h-R3	82.80 \pm 2.95	5.84 \pm 1.09	11.36 \pm 3.91
RT	63.62 \pm 2.83*	24.74 \pm 4.49	11.64 \pm 1.67
h-R3+RT	84.42 \pm 3.28*	7.79 \pm 7.30	7.79 \pm 4.09

Notes: h-R3: cells pretreated with nimotuzumab for 24 hours; RT: cells receiving 6 Gy radiation; h-R3+RT: cells pretreated with nimotuzumab and receiving 6 Gy radiation. Data was in the form of Mean \pm SD. *Indicates $P<0.05$.

Abbreviations: h-R3, nimotuzumab group; RT, irradiation group.

Table 3 Cell cycle distribution in H1975 cells ($\bar{x} \pm s$)

	G0/G1 (%)	G2/M (%)	S phase (%)
Control	89.60 \pm 3.31	1.92 \pm 2.04	8.48 \pm 1.26
h-R3	90.45 \pm 1.42	2.93 \pm 1.15	6.62 \pm 2.45
RT	73.00 \pm 5.95	17.55 \pm 7.47	9.45 \pm 1.66
h-R3+RT	78.29 \pm 3.07	13.03 \pm 2.87	8.68 \pm 0.20

Notes: h-R3: cells pretreated with nimotuzumab for 24 hours; RT: cells receiving 6 Gy radiation; h-R3+RT: cells pretreated with nimotuzumab and receiving 6 Gy radiation. Data was in the form of Mean \pm SD.

Abbreviations: h-R3, nimotuzumab group; RT, irradiation group.

the nucleus, EGFR and p-EGFR expression were suppressed in both cell lines; however, the decrease was more significant in H292 cells ($P<0.05$, Figure 3).

Pretreatment with nimotuzumab decreased nuclear phosphorylated DNA-PK (p-DNA-PK) expression in response to radiation in H292 cells

DNA-PK was involved in DNA damage repair. Nimotuzumab combined with radiation suppressed H292 p-DNA-PK expression in both the cytoplasm and the nucleus. There was a significant difference in the nuclear decrease ($P<0.05$, Figure 4); however, H1975 p-DNA-PK remained nearly the same in both the cytoplasm and the nucleus.

Discussion

More than 50% of cancer patients will receive radiotherapy during their treatment course.¹² Radioresistance is one of the obstacles that must be conquered to increase radiosensitivity. Nimotuzumab has demonstrated the effect of radiosensitization in various carcinomas, such as head and neck cancer, glioma, and esophageal cancer, and has been granted approval for use in such indications in different countries.^{13–16} Nonetheless, research regarding nimotuzumab's ability to promote radiosensitivity in NSCLC remains limited.^{9,17,18} Our research focused on the nimotuzumab radiosensitivity difference between NSCLC cell lines H292 and H1975, and explored the potential mechanism. The results showed that nimotuzumab inhibited H292 cells proliferation in a time- and dose-dependent manner, but it scarcely affected the proliferation of H1975 cells. The reason for this might be that nimotuzumab binded to cells with high EGFR expression, for example, H292 cells, in the form of bivalent combination that was more stable, but for cells with lower EGFR expression, such as H1975 cells, the combination tended to transient and monovalent. The stable bivalent combination between nimotuzumab and H292 cells thus inhibited EGFR signaling transduction and suppressed proliferation.¹⁹

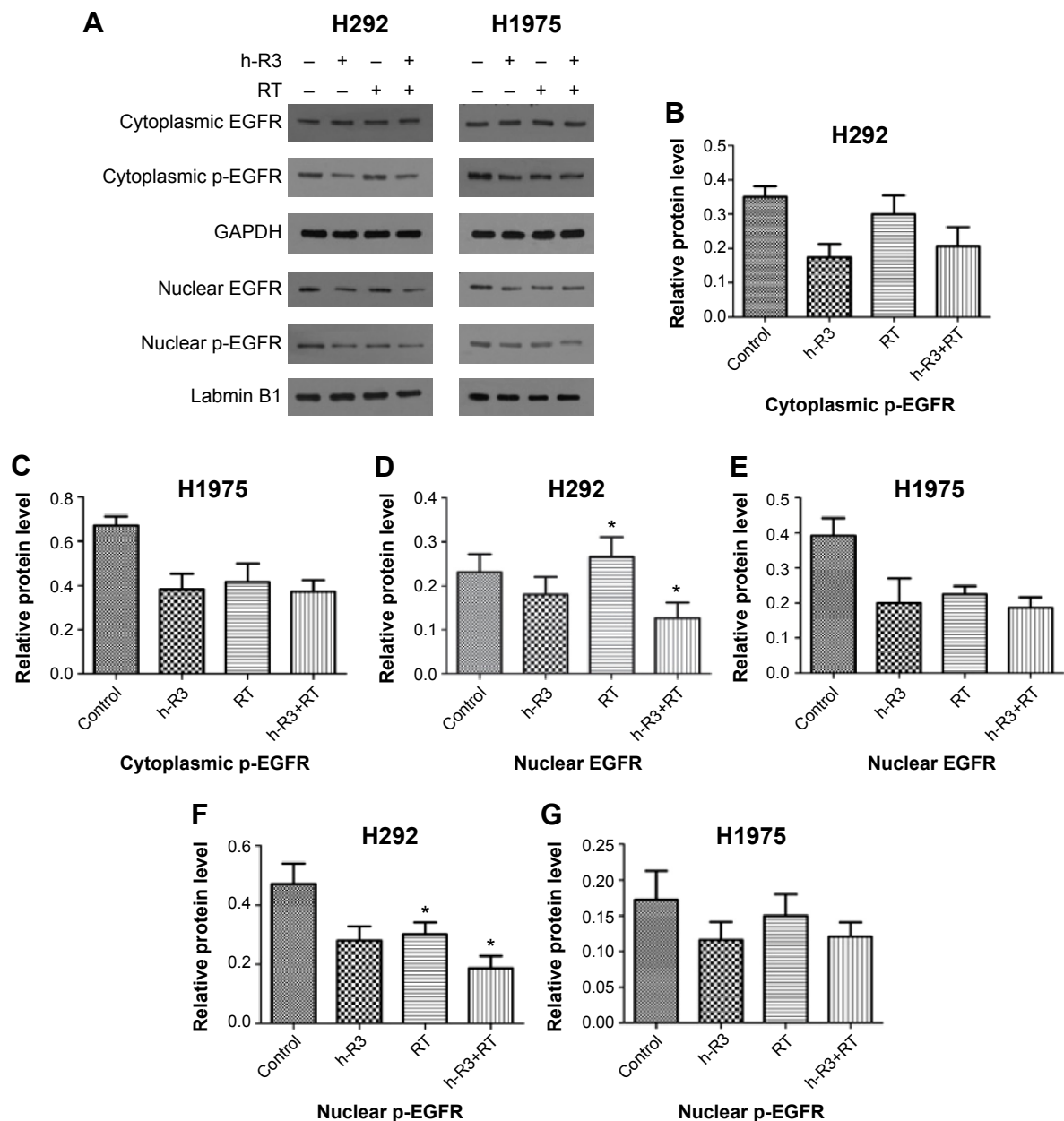


Figure 3 Illustration of EGFR/p-EGFR expression in both cytoplasm and nucleus.

Notes: Cytoplasmic p-EGFR expression was decreased in both cell lines when comparing the RT group with the RT+h-R3 group. The decrease was more significant in H292 cells, but the decrease did not reach statistical significance (**B** and **C**). Nuclear EGFR and p-EGFR was significantly decreased in H292 cells (**D** and **F**, $P < 0.05$), and the decrease in H1975 cells was not statistically significant (**E** and **G**). (**A**) illustrates the brief profile of protein expression ($n=3$). Each bar represents the mean \pm SD and *indicates significant difference ($P < 0.05$).

Abbreviations: EGFR, epidermal growth factor receptor; p-EGFR, phospho-epidermal growth factor receptor; h-R3, nimotuzumab group; RT, irradiation group.

Nimotuzumab arrested H292 cell cycle at the G0/G1 phase when combined with irradiation, but it hardly influenced H1975 cell cycle distribution. Cells in the G0/G1 phase are relatively sensitive to radiation, and this can partially explain the radiosensitivity enhancement in H292 cells.

Previous research showed that the EGFR inhibitor promoted cell apoptosis and increased radiation sensitivity.^{20–22}

Our apoptosis results demonstrated that there was no significant difference in either cells, although H292 cells exhibited a trend of higher apoptotic percentage compared with H1975 cells. In addition to apoptosis, other mechanisms such as autophagy and senescence are also involved. Growth factor-deprived cells maintained their metabolism through autophagy. Cetuximab downregulated HIF- α and Bcl-2 and induced autophagy in cancer cells.^{23,24} The research on esophageal

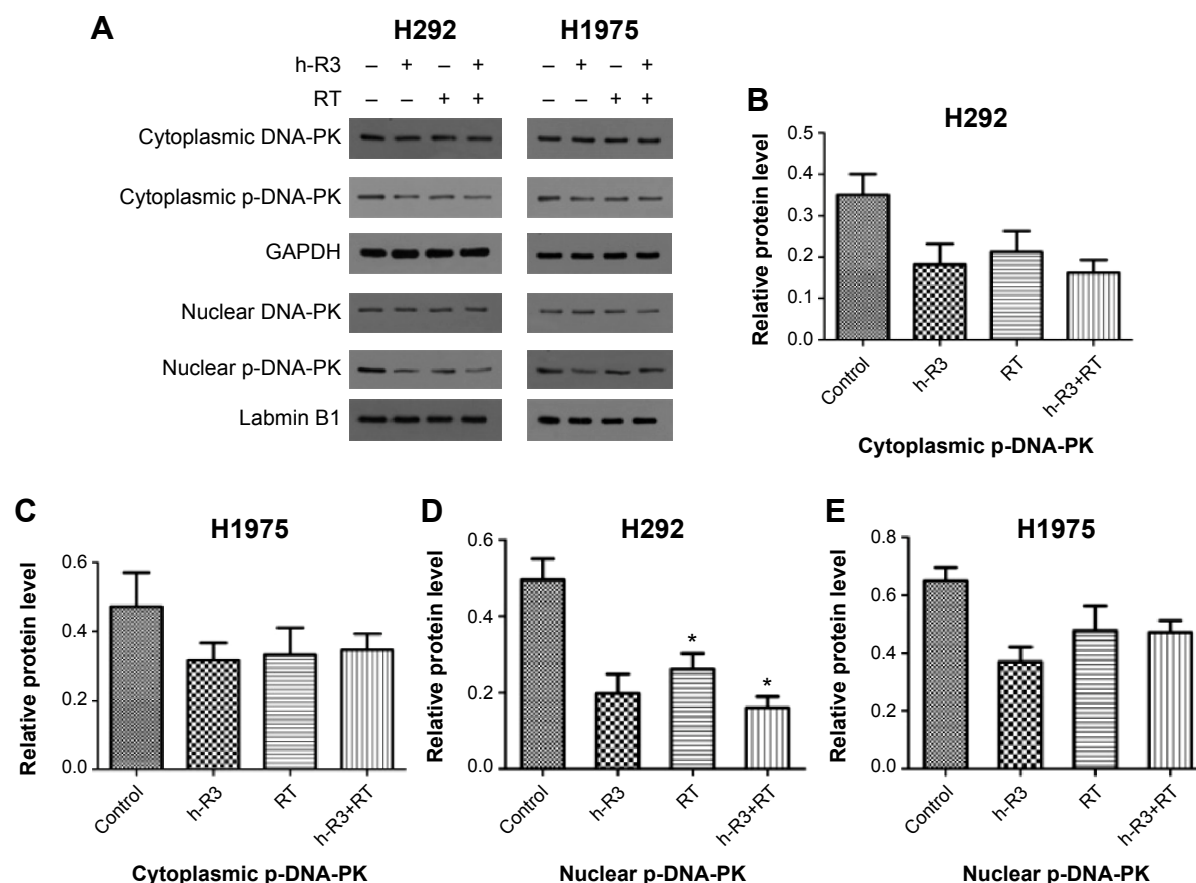


Figure 4 DNA damage repair after irradiation with or without nimotuzumab.

Notes: The p-DNA-PK expression was related to the DNA damage repair activity. Nimotuzumab suppressed H292 cytoplasmic p-DNA-PK expression and scarcely influenced p-DNA-PK expression in H1975 cells (**B** and **C**). Nuclear p-DNA-PK in H292 cells was suppressed ($P < 0.05$), while it almost remained the same in H1975 cells (**D** and **E**). (**A**) illustrates the brief profile of protein expression ($n=3$). Each bar represents the mean \pm SD and *indicates significant difference ($P < 0.05$).

Abbreviations: p-DNA-PK, phosphorylated DNA-PK; h-R3, nimotuzumab group; RT, irradiation group.

carcinoma found that nimotuzumab activated autophagy in Eca-109 cell with high EGFR expression without increasing cell apoptosis.²⁵ Wang et al demonstrated that EGFR inhibitors such as cetuximab or erlotinib increased radiosensitivity through senescence but not apoptosis.²⁶ It is still unknown to what extent apoptosis played a role in the radiation sensitivity of nimotuzumab for these two NSCLC cell lines; this is an area that will require further exploration.

The most detrimental DNA damage after ionizing irradiation is the DSB, which is preferentially repaired by nonhomologous end-joining in mammals.²⁷ H2AX is a histone H2A variant that constitutes 2%–25% of mammalian histone H2A depending on the organism and cell type.²⁸ γ -H2AX is the serine amino acid of H2AX phosphorylated at position 139 in response to DNA damage. The protein level reveals the severity of the damage. It is associated with the occurrence of DSB and often serves as a DNA damage indicator.

DNA-PK is a critical protein involved in nonhomologous end-joining repair of DSB, and is composed of DNA-PKcs and

two subunits Ku70 and Ku80. EGFR is phosphorylated upon irradiation. On one hand, it initiated the PI3K/AKT pathway and AKT translocated into the nucleus and recruited nuclear DNA-PK. Nimotuzumab blocked the PI3K/AKT pathway and inhibited the radiation-induced DNA-PK, and this was a possible mechanism for radiosensitivity enhancement.^{10,21} On the other hand, EGFR translocated into the nucleus and formed DNA end-binding protein complexes together with relative proteins, such as DNA-PK. Cetuximab inhibited EGFR nuclear translocation, reduced nuclear DNA-PK, and increased radiosensitivity.^{10,11} To our knowledge, there has been no published study on the effects of nimotuzumab on EGFR nuclear translocation and DSB repair. Our research initially explored the relationship of nimotuzumab and EGFR nuclear translocation in response to radiation. Nimotuzumab decreased the EGFR/p-EGFR nuclear translocation in H292 cells more significantly than it did in H1975 cells, and it reduced the nuclear p-DNA-PK expression in H292 cells simultaneously. One possible reason is that nimotuzumab inhibited the EGFR

pathway and suppressed EGFR translocation and nuclear DNA-PK phosphorylation. The DNA repair mechanism was impaired in such circumstances with no sufficient nuclear p-EGFR and p-DNA-PK to form the DNA end-binding protein complex, and thus increased radiosensitivity.

Nimotuzumab is a distinct EGFR monoclonal antibody different from familiar cetuximab. It binds to domain III of the extracellular region of the EGFR that overlaps with both the surface patch recognized by cetuximab and the binding site for ligand, such as EGF. Nimotuzumab blocks EGF binding but it still allows EGFR to adopt its active conformation and warrants a basal level of signaling.^{29,30} Moreover, the KD values that reflect the combination affinity for cetuximab and nimotuzumab are 2.3×10^{-9} M and 2.1×10^{-8} M, respectively. The affinity of cetuximab is ten times higher than that in nimotuzumab, justifying the lower rash incidence reported from nimotuzumab in clinical use.^{31,32} It also explains why we continued our research in nimotuzumab.

Of course, our primary results revealed the radiosensitivity influence of nimotuzumab from just one time point. More time points are needed to further understand the detailed relationship between nimotuzumab and nuclear EGFR translocation. We have a long way to go to make the mechanism clear and in the near future, to select the right person for the right regimen translationally.

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Disclosure

The authors report no conflicts of interest in this work.

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