

## Combination Effect of Nimotuzumab with Radiation in Colorectal Cancer Cells

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**Purpose:** To investigate the radiosensitizing effect of the selective epidermal growth factor receptor (EGFR) inhibitor nimotuzumab in human colorectal cancer cell lines.

**Materials and Methods:** Four human colorectal cancer cell lines, HCT-8, LoVo, WiDr, and HCT-116 were treated with nimotuzumab and/or radiation. The effects on cell proliferation, viability, and cell cycle progression were measured by MTT, clonogenic survival assay, flow cytometry, and Western blot.

**Results:** An immunoblot analysis revealed that EGFR phosphorylation was inhibited by nimotuzumab in colorectal cancer cell lines. Under these experimental conditions, pre-treatment with nimotuzumab increased radiosensitivity of colorectal cancer cell lines, except for cell line HCT-116. However, cell proliferation or cell cycle progression was not affected by the addition of nimotuzumab, irrespective of irradiation.

**Conclusion:** Nimotuzumab enhanced the radiosensitivity of colorectal cancer cells *in vitro* by inhibiting EGFR-mediated cell survival signaling. This study provided a rationale for the clinical application of the selective EGFR inhibitor, nimotuzumab in combination with radiation in colorectal cancer cells.

**Key Words:** Colorectal cancer, EGFR, Nimotuzumab, Radiation, Combined therapy

### Introduction

Epidermal growth factor receptor (EGFR) is overexpressed in approximately one-third of all human cancers, and has been directly implicated in tumor growth and progression.<sup>1)</sup> EGFR is one of the most important growth factor receptors, and has a critical role in regulation of cell proliferation, differentiation, angiogenesis, metastasis, and tumor invasiveness.<sup>2)</sup> Colorectal

cancer in particular is frequently associated with a high level of EGFR expression, resulting in advanced disease and a poor prognosis.<sup>3)</sup> Therefore, EGFR has been considered as an important target of cancer therapy, and blockade of EGFR may be a useful strategy for treatment of colorectal cancer.

Radiotherapy aims to reduce local recurrence and improve survival for colorectal cancer patients.<sup>4)</sup> However, radiation-induced release of TGF- $\alpha$  activates EGFR and mitogen-activated protein kinase (MAPK) pathways in carcinoma cells, leading to increased cell proliferation and protection from radiation-induced cell death.<sup>5)</sup> This relationship has important implications for treatment strategies designed to control EGFR-expressing cancer cells that survive after radiation exposure. Several studies have been undertaken to determine whether or not blockade of EGFR signaling can improve radiation cytotoxicity in lung and head and neck cancer.<sup>6~10)</sup>

Several agents, including small molecule inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI) and monoclonal antibodies (mAbs) specific for EGFR, have been designed for selective blockade of EGFR signaling.<sup>11,12)</sup> A humanized anti-EGFR mAb known as nimotuzumab (also known as h-R3),

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which binds to the extracellular domain of EGFR and inhibits EGF binding, was recently developed.<sup>13)</sup> Nimotuzumab has been approved in several countries for treatment of head and neck cancer<sup>14)</sup> and glioma,<sup>15)</sup> and is currently undergoing clinical evaluation for various tumor types, including colorectal, pancreatic, prostate, non-small cell lung, esophageal, cervical, and breast cancer.<sup>16)</sup> In a preclinical study, nimotuzumab demonstrated G<sub>0</sub>/G<sub>1</sub> cell cycle arrest, proapoptotic and antiangiogenic activity against an EGFR-overexpressing A431 cell line,<sup>17)</sup> and enhanced antitumor efficacy of radiation in certain human non-small cell lung cancer cell lines *in vitro* and *in vivo*.<sup>18)</sup> Moreover, a phase I/II trial showed that nimotuzumab was well tolerated and enhanced the curative potential of radiation in patients with advanced head and neck cancer.<sup>14)</sup>

In regard of colorectal cancer, however, there are no published reports on the therapeutic effect of nimotuzumab, a recently developed humanized EGFR inhibitor. Therefore, we speculated that blockade of EGFR signaling by addition of nimotuzumab to radiotherapy may possibly enhance therapeutic efficacy for colorectal cancer patients. To answer this question, we first investigated the radiosensitizing effects of nimotuzumab in several colorectal cancer cell lines *in vitro*.

## Materials and Methods

### 1. Material

Nimotuzumab was obtained from YM BioSciences (Mississauga, ON, Canada) at a concentration of 5 mg/mL. All other chemicals and proteins not specified were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2. Cell lines and cultures

The human colon adenocarcinoma cell line HCT-8 was obtained from the American Type Culture Collection (Manassas, VA, USA) and was maintained in RPMI-1640 (WelGene, Daegu, Korea) containing 10% horse serum (Gibco, Grand Island, NY, USA). LoVo, WiDr, and HCT-116 were obtained from the Korean Cell Line Bank (Seoul, Korea). The LoVo and HCT-116 cell lines were maintained in RPMI-1640, and the WiDr cell line was maintained in DMEM, supplemented with 10% fetal bovine serum (WelGene). All cells were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

### 3. *In vitro* cell growth

A total of 3 to 4×10<sup>5</sup> exponentially growing cancer cells were seeded in 35 mm plates (SPL, Seoul, Korea) and incubated in growth medium overnight at 37°C. Nimotuzumab (500 nM) was added 4 hours prior to radiation exposure; incubation was then continued for 48 hours at 37°C. Gamma radiation was delivered using a dual-source <sup>137</sup>Cs unit at a dose rate of 3.2 Gy/minutes with a GC-3000 Elan irradiator (MDS Nordion, Ottawa, ON, Canada). Five mg/mL of MTT solution was added to each dish for 2 hours at 37°C to allow formation of MTT formazan crystals by metabolically active cells. Formazan crystals were dissolved in DMSO and plates were shaken for 30 minutes at room temperature. Absorbance of each well was measured using a microplate reader at a wavelength of 540 nm.

### 4. Clonogenic survival assay

Clonogenic survival was defined as the ability of cells to maintain clonogenic capacity and to form colonies.<sup>19)</sup> Briefly, cells were harvested from exponential phase cultures by trypsinization, counted, and seeded for colony formation. Following incubation overnight, 500 nM nimotuzumab was added for 4 hours prior to radiation exposure. After 14~20 days, colonies were stained with 0.4% crystal violet. Colony counts were determined using a counter; a count of >30 cells was considered a colony. Plating efficiency (PE) represents the percentage of cells seeded that actually grow into colonies under the specific culture conditions of a given cell line. The survival fraction, expressed as a function of irradiation, was calculated as follows: survival fraction=colonies counted/(cells seeded×PE/100).

### 5. Flow cytometry for cell cycle analysis

Nimotuzumab (500 nM) was added 4 hours prior to radiation exposure; incubation was then continued for 24 hours at 37°C. Cells were washed with PBS and harvested by trypsinization. After centrifugation, cell pellets were fixed in cold 70% ethanol. After washing in PBS, cells were subsequently stained with a DNA staining solution (20 μg/mL of propidium iodide and 10 μg/mL of RNase A) for 30 minutes. Stained cells were then suspended and immediately subjected to analysis by flow cytometry (BD Biosciences, San Jose, CA,

USA). Ten thousand cells in each sample were analyzed, and cell cycle distribution was calculated using Cell Quest software (BD Biosciences).

### 6. Western blot analysis

Serum starved cells were exposed to nimotuzumab (500 nM) for 4 hours and then treated with 50 nM of EGF for 5 minutes. Cells were lysed with SDS sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 15% glycerol, and 0.004% bromophenol blue) and were then boiled for 10 minutes. Twenty microgram of cell lysate were separated by 8% to 10% SDS-polyacrylamide gels, transferred to a nitrocellulose membrane, and immunoblotted and detected by chemiluminescence using ECL detection reagents. Anti- $\alpha$ -tubulin antibody was purchased from Santa Cruz (Santa Cruz, CA, USA) and all other antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

### 7. Statistical analysis

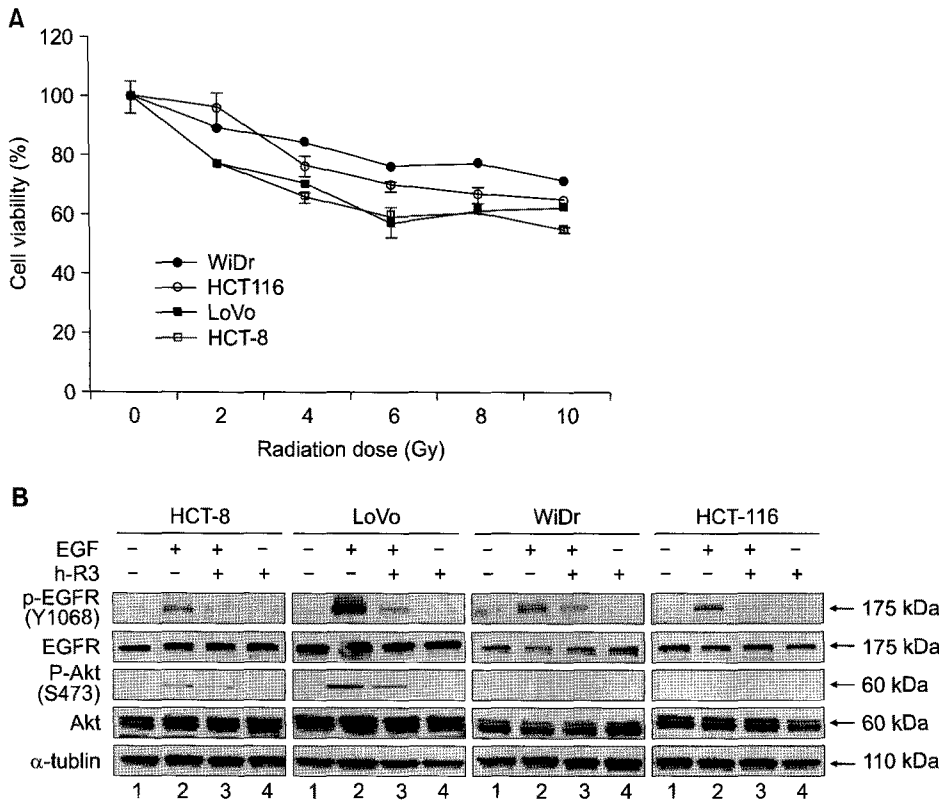
*In vitro* results were analyzed using the Tukey's Honestly Significant Differences Test and independent-sample t-test with

SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA) and p-values <0.05 were accepted as statistically significant.

## Results

### 1. Effect of nimotuzumab on EGFR signaling

To evaluate the combination effect of nimotuzumab and radiation, four human EGFR expressing colorectal cancer cell lines, HCT-8, LoVo, WiDr, and HCT-116 were selected. First, the optimal dose of radiation was determined that caused approximately 20% growth inhibition of each type of colorectal cancer cells using MTT assay. Therefore suitable radiation dose of each cell line was determined as 6 Gy for WiDr and 4 Gy for HCT-116 and 2 Gy for LoVo and HCT-8 (Fig. 1A). Next, we confirmed that 500 nM of nimotuzumab sufficiently inhibited the EGF-induced EGFR phosphorylation in these colorectal cancer cell lines. Serum-starved colorectal cancer cells were stimulated with EGF; EGF-mediated EGFR autophosphorylation and downstream Akt activation were then examined by Western blot analysis (Fig. 1B). Phosphorylation of EGFR was undetectable in serum-starved cells (lane 1), but

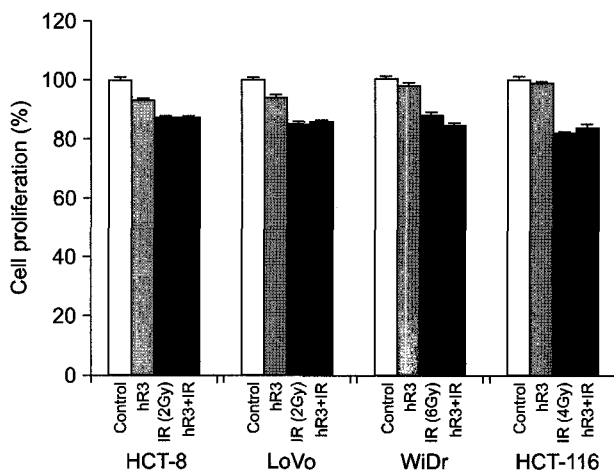


**Fig. 1.** Effect of irradiation on cell viability and inhibition of epidermal growth factor (EGF)-induced epidermal growth factor receptor (EGFR) signaling by nimotuzumab in colorectal cancer cell lines. (A) Sensitivity of colon cancer cell lines to different doses of radiation was analyzed using MTT assay. Cells were treated with radiation on day 0 and incubated further for 48 hours. Values are means±standard deviation from triplicates for each point. (B) Phosphorylation of EGFR and Akt in human colorectal cancer cells was examined after EGF (50 ng/mL) stimulation for 10 min with or without pretreatment of nimotuzumab (500 nM) for 4 hours. Protein extracts were quantified and equal amounts of total protein were subjected to SDS-PAGE. Immunoblot analysis was performed with the indicated antibodies.

was markedly induced on exposure of cells to EGF (lane 2). EGF-induced phosphorylation of EGFR in these cells was clearly inhibited by 500 nM of nimotuzumab (lane 3). Downstream Akt phosphorylation might be inhibited by nimotuzumab in HCT-8 or LoVo cells, however, no significant phosphorylation of Akt was observed in the WiDr or HCT-116 cells. These results indicate that nimotuzumab bound EGFR and blocked EGF-mediated EGFR signaling in colorectal cancer cell lines.

## 2. Effect of nimotuzumab in combination with radiation on cell proliferation

Under the selected experimental condition, the combined effect on cell proliferation was then assessed using the MTT assay 48 hours after exposure to nimotuzumab, radiation, or both. As shown in Fig. 2, the effect of nimotuzumab on cell proliferation was insignificant among the four colorectal cancer cell lines. Although a combination of nimotuzumab and radiation inhibited cellular proliferation by approximately 20% for all colorectal cancer cell lines, these results were similar to results of radiation alone. According to these results, inhibition of EGFR signaling by nimotuzumab did not inhibit colorectal cancer cell proliferation either combined with radiation or alone.



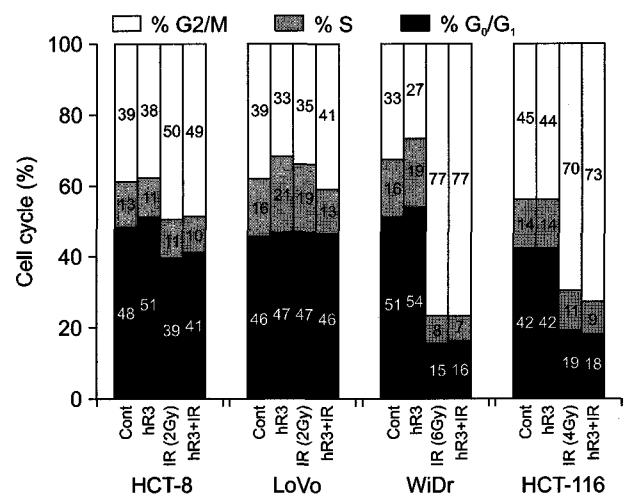
**Fig. 2.** Effect of nimotuzumab combined with radiation on colorectal cancer cell proliferation. Cells were treated with nimotuzumab (500 nM) for 4 hours prior to irradiation. After 48 hours, cell proliferation was analyzed using the MTT assay. All treated cells were normalized to respective untreated controls. Values are means±standard deviation for triplicate wells for each dose point. hR3: nimotuzumab, IR: ionizing radiation.

## 3. Effects of nimotuzumab, radiation, and combination exposure on cell cycle distribution in colorectal cancer cell lines

As nimotuzumab demonstrated G<sub>0</sub>/G<sub>1</sub> cell cycle arrest in EGFR-overexpressing A431 cell line previously,<sup>17)</sup> the effect of nimotuzumab and/or radiation on cell cycle distribution was assessed in colorectal cancer cell lines after 24 hours of exposure. In colorectal cancer cell lines, however, the cell cycle showed no change in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle with nimotuzumab treatment compared with control (Fig. 3). Although cell cycle arrest in G<sub>2</sub>/M phase by radiation was obvious in WiDr and HCT-116 cells, nimotuzumab combined with radiation also showed a similar increase in the G<sub>2</sub>/M fraction. This result indicated that nimotuzumab did not induce G<sub>0</sub>/G<sub>1</sub> arrest or any significant changes in cell cycle progression in colorectal cancer cell lines, irrespective of radiation.

## 4. Effect of nimotuzumab on radiosensitivity in colorectal cancer cell lines

In order to determine the effect of nimotuzumab on radiosensitivity of colorectal cancer cells, clonogenic survival analysis was performed. Statistical analysis of survival curves

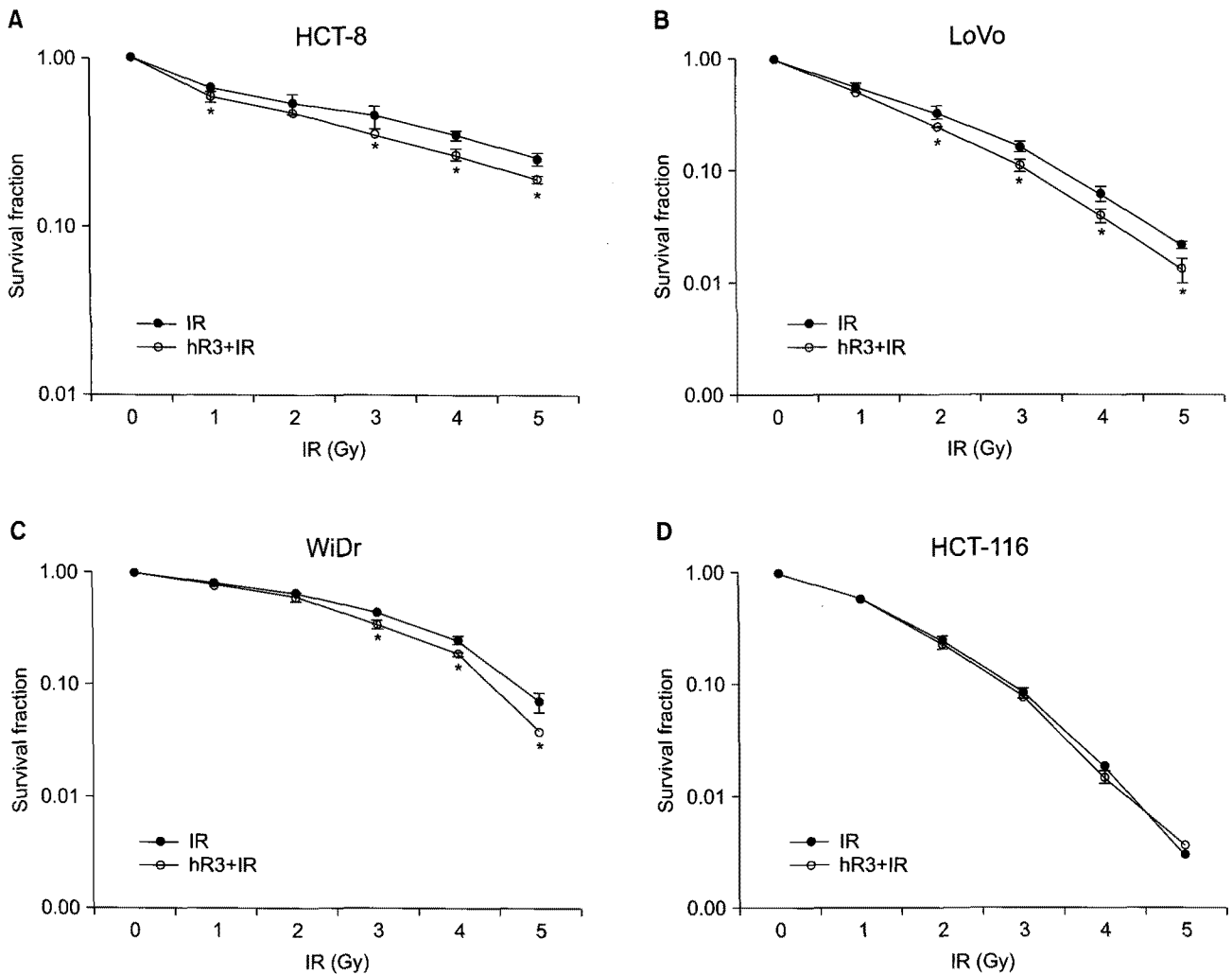


**Fig. 3.** Effects of nimotuzumab, radiation, and combination exposure on cell cycle distribution in colon cancer cell lines. Cells were irradiated in the absence or presence of nimotuzumab (500 nM) for 4 hours. After 24 hours, PI staining cells were analyzed by FACS. Values are means±standard deviation for triplicate wells for each dose point. hR3: nimotuzumab, IR: ionizing radiation.

revealed that the cytotoxic effect of radiation on HCT-8, LoVo, and WiDr cells was enhanced significantly, with dose enhancement factors of 1.33, 1.67, and 1.86, respectively ( $p < 0.05$ ) (Fig. 4). However, different from results of the other cell lines, treatment with nimotuzumab had no effect on radiation sensitivity of HCT-116 cells, with a dose enhancement factor of 0.83. Results from clonogenic survival assays indicate that combination of nimotuzumab enhanced the radiosensitivity of most of colorectal cancer cells, although cell type-dependent effect could not be ruled out completely.

## Discussion and Conclusion

In the application of monoclonal antibodies (mAbs) to oncology, the most recent and most significant advances have been the introduction and approval of cetuximab (Erbix) and panitumumab (Vectibix), anti-epidermal growth factor antibodies. In addition, numerous antibodies, such as matuzumab and nimotuzumab, and tyrosine kinase inhibitors, such as gefitinib, erlotinib, lapatinib, and canertinib, which target EGFR, are in clinical development or under preclinical investigation.<sup>20</sup> In combination with standard chemotherapy regimens, cetuximab in particular, used alone or with radio-



**Fig. 4.** Effect of nimotuzumab on intrinsic radiosensitivity. A clonogenic assay was performed after exposure to the indicated dose of radiation in the absence or presence of nimotuzumab (500 nM) in the colorectal cancer cell lines HCT-8 (A), LoVo (B), WiDr (C), and HCT-116 (D). Asterisk \* represents a statistically significant difference ( $p < 0.05$ ) between control cells and nimotuzumab-combined cells. hR3: nimotuzumab, IR: ionizing radiation.

therapy, produces clinically meaningful anti-tumor responses in patients with chemotherapy-refractory colorectal cancer.<sup>21)</sup> Also, in our previous study, cetuximab proved to have synergistic cytotoxic effects when combined with radiation in preclinical tumor models.<sup>22)</sup> These recent exciting results provide optimism for development of mAbs that bind EGFR, exploit novel mechanisms of action, or possess improved tumor targeting.

It has recently been proposed that skin toxicity is a surrogate marker of EGFR blockade, given the association between survival and degree of rash in patients treated with several EGFR inhibitors.<sup>23)</sup> However, skin rash was not seen after repeated doses of nimotuzumab, and after 6 doses of nimotuzumab, only 1 patient developed an immune response against the humanized molecule, with no deleterious clinical effect upon the patient. This may be related to the fact that nimotuzumab is a humanized antibody with a larger proportion of human sequence, and could activate immune effector cells in the tumor area more efficiently than murine mAbs, such as cetuximab.<sup>14,24,25)</sup> Moreover, radiosensitization of head and neck cancer cells mediated by nimotuzumab is supported by preclinical and clinical studies.<sup>14,26)</sup> To better understand the influence of the EGFR blockade on radiation response in colorectal cancer, we investigated the antitumor effect of combined treatment with nimotuzumab and radiation in colorectal cancer cell lines. In this study, nimotuzumab inhibited EGF-induced phosphorylation of EGFR and Akt in colorectal cancer cell lines (Fig. 1B) and significantly enhance radiosensitivity when combined with radiation in most of colorectal cancer cell lines (Fig. 4). Interestingly, clonogenic survival assay showed that nimotuzumab enhanced radiosensitivity of WiDr cell which was resistant to cetuximab treatment.<sup>22)</sup> In contrast to the results using cetuximab,<sup>22)</sup> nimotuzumab neither inhibited the cell proliferation irrespective of irradiation (Fig. 2) nor caused cell cycle arrest in G<sub>0</sub>/G<sub>1</sub> phase (Fig. 3). It is notable that not only suppressing the EGFR-mediated cell survival signaling, there could be additional radiosensitizing mechanisms of nimotuzumab. Systemic effects of nimotuzumab also showed promising results in other cancer type. Nimotuzumab inhibited tumor invasion, suppressed angiogenesis and targeted CD133<sup>+</sup> cancer stem cell population, which eventually led to inhibition of tumor growth *in vivo*.<sup>26)</sup> To verify these host-related therapeutic effects of nimotu-

zumab on colorectal cancer cell, *in vivo* animal study is required.

In summary, we have shown that nimotuzumab enhanced the antitumor efficacy of radiation *in vitro*, providing a rationale for future clinical investigations of the therapeutic efficacy of nimotuzumab in combination with radiotherapy.

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## 대장암 세포에서 EGFR 저해제 Nimotuzumab의 방사선 병합 효과

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**목적:** 대장암 세포에서 epidermal growth factor receptor (EGFR) 저해제인 nimotuzumab에 의한 방사선 민감도 증진 효과를 살펴보고자 한다.

**대상 및 방법:** 총 4종류의 인간 유래 대장암 세포주인 HCT-8, LoVo, WiDr, HCT-116를 nimotuzumab과 방사선을 병합 처리한 후 세포증식, 생존율, 세포주기 진행에 미치는 영향을 MTT, clonogenic survival assay, flow cytometry와 western blot을 통해 분석하였다.

**결과:** 대장암 세포주에서 nimotuzumab에 의해 EGFR 인산화가 억제됨을 확인하였고 이러한 조건에서 nimotuzumab이 HCT-116을 제외한 나머지 3종류의 대장암 세포주의 방사선 민감도를 증진시킴을 확인하였다. 반면에, nimotuzumab은 방사선 조사와 무관하게 대장암 세포의 증식이나 세포 주기에는 아무런 영향을 미치지 않았다.

**결론:** Nimotuzumab은 EGFR에 의한 세포 생존 신호 전달을 억제함으로써 대장암 세포의 방사선에 대한 민감도를 증가시켰다. 본 연구는 대장암의 방사선 치료에 EGFR 특이적 저해제인 nimotuzumab의 임상 적용 근거를 제공하였다.

**핵심용어:** 대장암, EGFR, Nimotuzimab, 방사선, 병합치료