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Original Article

Relationship between uptake of a radioiodinated quinazoline derivative and radiosensitivity in non-small cell lung cancer

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Abstract: The aim of this study was to determine whether the uptake of radioiodinated 6-(3-morpholinopropoxy)-7-ethoxy-4-(3'-iodophenoxy) quinazoline ([^{125}I]PYK) could predict the response of non-small cell lung cancer (NSCLC) cells to radiotherapy *in vitro*. Four NSCLC cell lines, PC9, HCC827, A549, and H1975 were used. Cells were irradiated with doses ranging from 2 Gy to 8 Gy and/or exposed to 1 μM gefitinib. The effects of radiation and gefitinib were assessed by the CCK-8 assay and confirmed by reverse transcription-polymerase chain reaction and immunoflorescence microscopy. The uptake of [^{125}I]PYK was determined by incubating cells with a tracer. The cell cycle was assessed by flow cytometry. The expression of EGFR was measured by western blotting. The results obtained revealed that the uptake of [^{125}I]PYK was higher in PC9 and HCC827 cells than in A549 and H1975 cells. PC9 cells and HCC827 cells were also more radiosensitive than A549 and H1975 cells. The gefitinib pretreatment reduced the S phase fraction and enhanced radiation effects in PC9 and HCC827 cells. These results indicate that the uptake of [^{125}I]PYK is related to the effects of radiation in NSCLC cells. Radioiodinated PYK may be useful in predicting the response of NSCLC in patients to radiotherapy.

Keywords: [^{125}I]PYK, irradiation, gefitinib, EGFR, NSCLC

Introduction

It is widely known that lung cancer is the leading cause of cancer-related death, and its incidence is increasing every year worldwide. Approximately 80% of lung cancers are non-small cell lung cancer (NSCLC) [1, 2]. Although several therapies have been developed in the last decade, the prognosis of advanced NSCLC remains poor with a 5-year survival rate of approximately 10-15% [3]. Molecular genetics has contributed greatly to studies of NSCLCs, such as alterations in protein kinase activation [4]. Epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor belonging to the ErbB family. EGFR controls many important cellular functions and plays an important role in the development and progression of many human malignancies [5-7]. In NSCLC, the overexpression of EGFR has been reported in 80% and approximately half of adenocarcinomas and large-cell carcinomas, respectively [8]. Abnormal alterations in the EGFR gene have been shown to play an important role in carcinogenic processes, metastasis, and a poor prognosis [9]. An EGFR tyrosine kinase inhibitor or gefitinib competitively binds to the adenosine triphosphate binding pocket in the kinase domain and blocks downstream signaling pathways [10]. However, gefitinib has exhibited antitumor effects in a subset of approximately 10% of NSCLC patients with specific EGFR gene mutations [1, 11]. Such mutations are more frequently found among women, non-smokers, patients with adenocarcinoma, and Japanese or East Asian patients [1, 12]. Molecular analyses identified two specific mutations that have been linked to gefitinib sensitivity: deletion mutations in exon 19 and a L858R mutation in exon 21 [7].

Previous studies suggested that the EGFR expression status may also be associated with radiosensitivity. NSCLC cell lines bearing the tyrosine kinase domain mutant of EGFR were found to be more sensitive to ionizing radiation

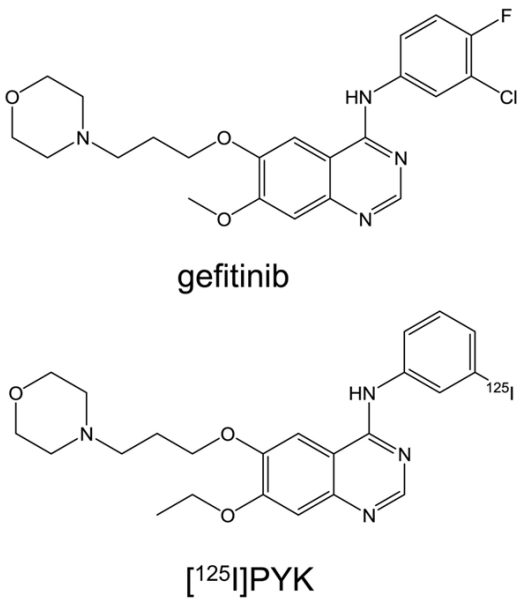

Figure 1. Chemical structures of gefitinib and [^{125}I] PYK.

than wild-type EGFR cell lines [13]. Furthermore, EGFR inhibitors have enhanced the effects of ionizing radiation [14].

Because the expression of and mutations in EGFR have been linked to the effectiveness of radiotherapy, the identification of a mutant EGFR gene is important to select patient subgroups in whether radiotherapy is more effective. Many methods have been developed for detecting EGFR gene mutations. In the past several years, a great deal of effort has been spent in developing non-invasive molecular imaging approaches to evaluate the EGFR status [15-17]. In a previous study, we reported the detection of a mutant EGFR gene with a newly developed EGFR tyrosine kinase imaging ligand, a radioiodinated 6-(3-morpholinopropoxy)-7-ethoxy-4-(3'-iodophenoxy) quinazoline ([^{125}I]PYK) [18, 19]. In the present study, we examined the relationship between the uptake of [^{125}I]PYK and the effects of radiation on lung cancer cells *in vitro*, and demonstrated that radiation was more effective in cells with high [^{125}I]PYK uptake than in cells with low [^{125}I]PYK uptake.

Materials and methods

Cells

To determine whether [^{125}I]PYK could specifically accumulate in EGFR-expressing tumors, we selected various tumor cell lines harboring different EGFR expression levels or mutational statuses. Four NSCLC cell lines, PC9, HCC827, A549, and H1975, were used. A549 was obtained from the Cell Resource Center for Medical Research at Tohoku University, and other cells were obtained from the American Type Culture Collection (Manassas, VA, USA). PC9 and HCC827 cells harbored an exon 19 mutation (DelE746A750) in the EGFR gene, H1975 harbored two missense mutations in EGFR (L858R, T790M), and A549 expressed wild type EGFR [20]. Cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and maintained at 37°C in a humidified 95% air - 5% CO_2 atmosphere. Cells were irradiated at room temperature.

Radiolabeling of PYK

[^{125}I]NaI was obtained from MP Biomedicals Japan (Tokyo, Japan). Unlabeled PYK was synthesized according the method described in a previous study [18]. [^{125}I]PYK was synthesized as follow: briefly, 10 μL of 30% aqueous hydrogen peroxide was added to a mixture of [^{125}I]NaI (10 μL, 37.0 MBq, 74 TBq/mmol), 0.1 M HCl (25 μL), and the tributylstannyl precursor of PYK (0.01 mg in 10 μL ethanol). After a 10-min reaction, [^{125}I]PYK was purified by HPLC. The radiochemical yield was 97.5%, and radiochemical purity was more than 95%. Chemical structures of gefitinib and [^{125}I]PYK were shown in **Figure 1**.

Radiation and gefitinib treatment

Cells were irradiated with a range of doses from 2 Gy to 8 Gy at a dose rate of 1.8 Gy/min by X-rays. Cells were exposed to 1 μM gefitinib (Cayman chemical, Ann Arbor, Michigan, USA) starting 24 hrs before irradiation and continuing throughout the experiment in the combined treatment.

Cell proliferation assay

After 48 hrs of radiation without or with the gefitinib treatment, the number of viable cells was quantified using a Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan). A total of 10 μL of CCK-8 solution was added to each well, and cells were then incubated for 2 hr at 37°C in 5% CO_2. Absorbance was measured at 450 nm using a microplate reader, according to the manufacturer's instructions.

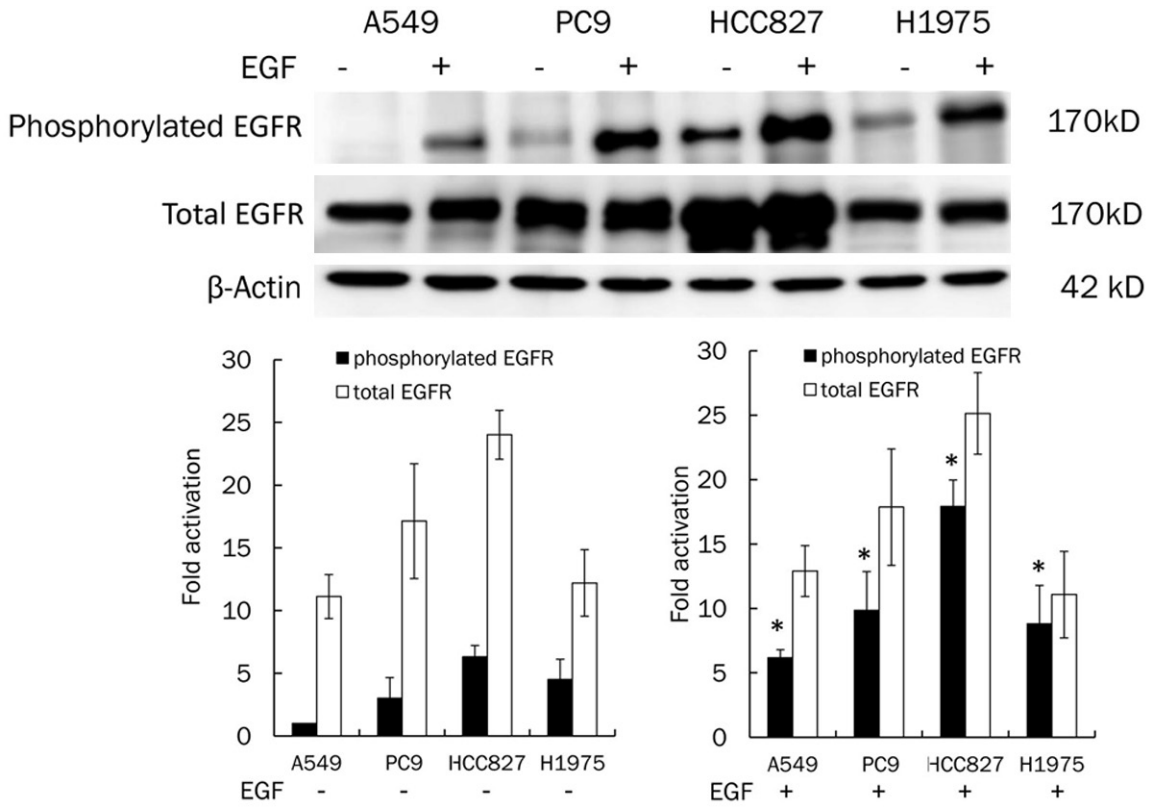

Figure 2. Western blotting analyses for EGFR expression on the cell surface. All 4 cell lines expressed a significant level of EGFR on the cell surface. β-actin was used as the loading control. Values are the mean ± SD from 4 independent experiments. *P<0.05 were obtained from the Student's *t*-test by comparing the expression level of phosphorylated EGFR measured with and without the EGF treatment in the same cell line.

Uptake of [^{125}I]PYK into cells

The binding of [^{125}I]PYK into cells was determined by a previously reported method [18]. Briefly, 0.74 kBq [^{125}I]PYK was added to 1.0×10^6 cells in 1 mL PBS. After a 2-hr incubation, the radioactivity incorporated into the cells was determined by a gamma counter. To test the specificity of binding, cells were pretreated with 10 μM unlabeled PYK or 10 μM EGFR kinase inhibitor PD153035 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 30 min at 37°C. A total of 0.74 kBq [^{125}I]PYK was then added to the cells.

EGFR expression

The expression of total EGFR and phosphorylated EGFR was analyzed using western blotting. Cancer cells were grown in 10-cm culture dishes until 60~70% confluent, and then cultured in medium without FBS for approximately 24 hrs for serum starvation. Cells were pretreated by EGF for 15 min and collected in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS with inhibitor cocktail (Sigma Aldrich, St Louis, MO, USA), and PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science, Basel, Switzerland). Extracted proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked overnight at 4°C with blocking buffer. Total EGFR was detected using a rabbit polyclonal antibody against the C-terminus of EGFR (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and phosphorylated EGFR was detected by a rabbit polyclonal antibody to phosphorylated EGFR at tyrosine 1068 (Cell Signaling Technology, Inc., Danvers, MA, USA) at room temperature for 2 hr. The membrane was washed in TBS-0.1% Tween 20. The primary antibody was detected using a horseradish peroxidase-conjugated secondary antibody for 1 hr and visualized with an ECL Prime Western Blotting Detection

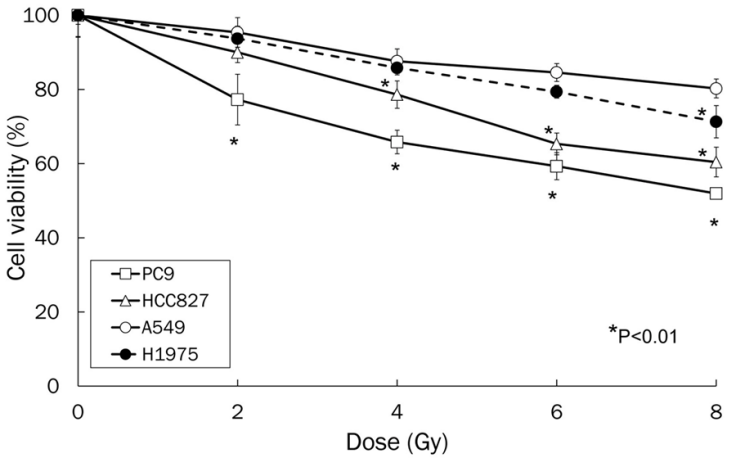

Figure 3. Decrease in the number of viable cells after irradiation as measured by the CCK-8 assay. Data represent the mean ± SD, n=4. PC9 and HCC827 cells were more radiosensitive than A549. No significant difference was observed between H1975 and A549 cells at 2, 4, and 6 Gy. **P* values from an ANOVA test were obtained by comparing A549 with PC9, HCC827, and H1975.

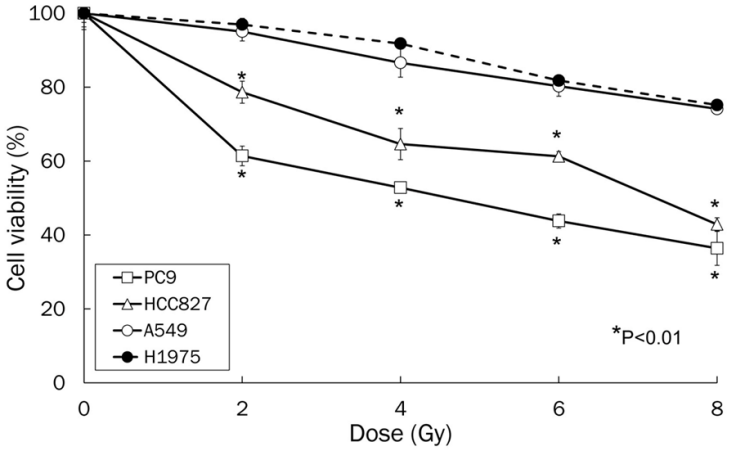

Figure 4. Decrease in the number of viable cells after irradiation with exposure to gefitinib as measured by the CCK-8 assay. Data represent the mean ± SD, n=4. Gefitinib enhanced the effects of radiation more in PC9 and HCC827 cells than in A549 and H1975 cells. **P* values from an ANOVA test were obtained by comparing A549 with PC9 and HCC827.

System (GE Healthcare, Buckinghamshire, UK). Bands were detected by LAS 3000 mini (Fujifilm, Tokyo, Japan). Band intensity was quantified by Image J software (National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Changes of EGFR mRNA expression were examined in the cells which were treated with 4 Gy X-ray radiation, 1 μM gefitinib exposure or both by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the cells by using ISOGEN reagent (Nippon Gene, Tokyo, Japan), and converted to first-strand cDNA by using a SuperScript First-Strand Synthesis System for reverse transcription (Invitrogen, Carlsbad, CA, USA) according to the supplier's protocol. RT-PCR was performed using EGFR primers 5'-CAATAACTGTGAGGTGGTCCTTGG-3' and 5'-CTCCTTCAGTCCGGTTTTATTTGC-3'. Beta-actin was used as an internal control with primers 5'-CATGTACGTTGCTATCCAGGC-3' and 5'-CTCCTTAATGTCACGCACGAT-3'. PCR products were fractionated by electrophoresis on a 2.0% agarose gel and stained with ethidium bromide, and the gel was examined under UV light. A 100-bp DNA ladder (New England Biolabs, Beverly, MA, USA) was used.

Immunofluorescence microscopy

Cells 24 hr after irradiation with a dose of 4 Gy or untreated cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. Permeabilization was performed with 0.1% Triton X-100 for 15 min at room temperature. Nonspecific staining was blocked by 1% BSA in PBS. Cells were incubated with rabbit anti-EGFR antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 1 hr. After washing, cells were incubated with Alexa 488-coupled goat-anti rabbit IgG antibody (Invitrogen, Carlsbad, CA, USA) for 1 hr. Nuclei were counterstained with 4',6-diamidine-2'-phenylindole-dihydrochloride (DAPI) (Vector Laboratories, Burlingame, CA, USA).

Cell cycle analysis

Changes in the cell cycle were analyzed after a 24-hr incubation with gefitinib. Cells were fixed with cold 70% (v/v) ethanol at -20°C overnight,

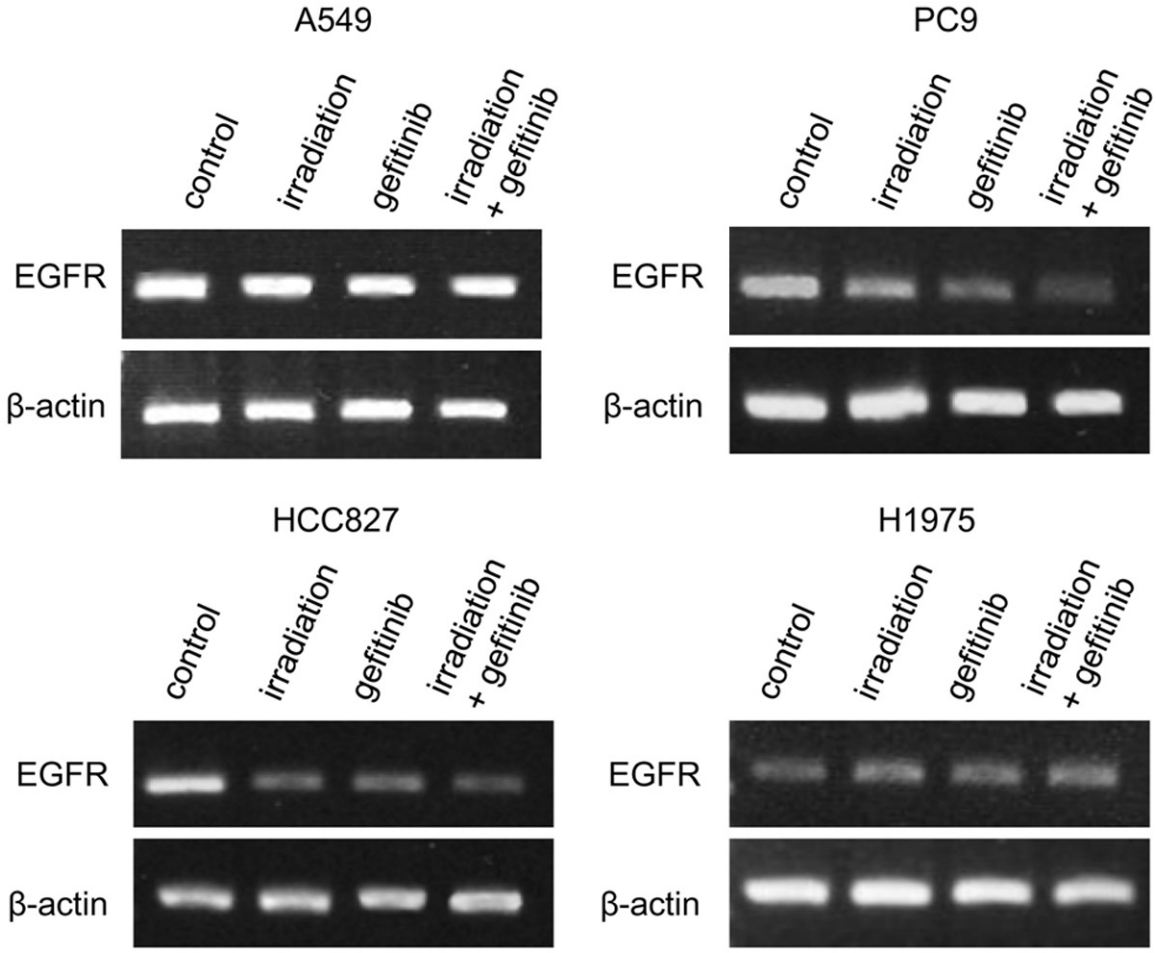

Figure 5. RT-PCR analysis of mRNA expression of EGFR in cells treated with irradiation, gefitinib and both. Irradiation and/or gefitinib treatment reduced mRNA expression of EGFR in HCC827 and PC9 cells but did not in A549 and H1975 cells.

permeabilized on ice in PBS-0.5% Triton X-100 for 15 min, washed, and resuspended in 0.5 mL of PBS containing 1% FBS. A total of 100 μL of RNase A (1 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was then added and, after 30 min of incubation at 37°C, the cells were stained with a solution containing 10 μg/mL of propidium iodide (PI) for 15 min (Sigma-Aldrich, St. Louis, MO, USA). Stained nuclei were analyzed for DNA-PI fluorescence using a flow cytometer (Beckman Coulter Inc. Brea, CA, USA). The resulting DNA distributions were analyzed by FlowJo (TreeStar Inc. Ashland OR, USA) for the proportion of cells in G1, S, and G2/M phases of the cell cycle.

Data analysis

Results were expressed as means ± standard deviation of mean. Comparisons between cells were performed by a one-way ANOVA followed by Tukey's multiple comparisons post hoc test with α set at $P<0.01$. Comparisons of data in the same cell line were performed using the Student's *t*-test. $P<0.05$ was considered significant. Statistical analysis was performed with SPSS 16.0J (International Business Machines Corp. Armonk, NY, USA).

Results

EGFR expression and activation status of EGFR

As shown in **Figure 2**, all 4 cell lines expressed a significant level of total EGFR. HCC827 showed the highest EGFR expression. With serum starvation, phosphorylated EGFR was nearly absent in A549 cells with Wt EGFR. However, the phosphorylation of EGFR was

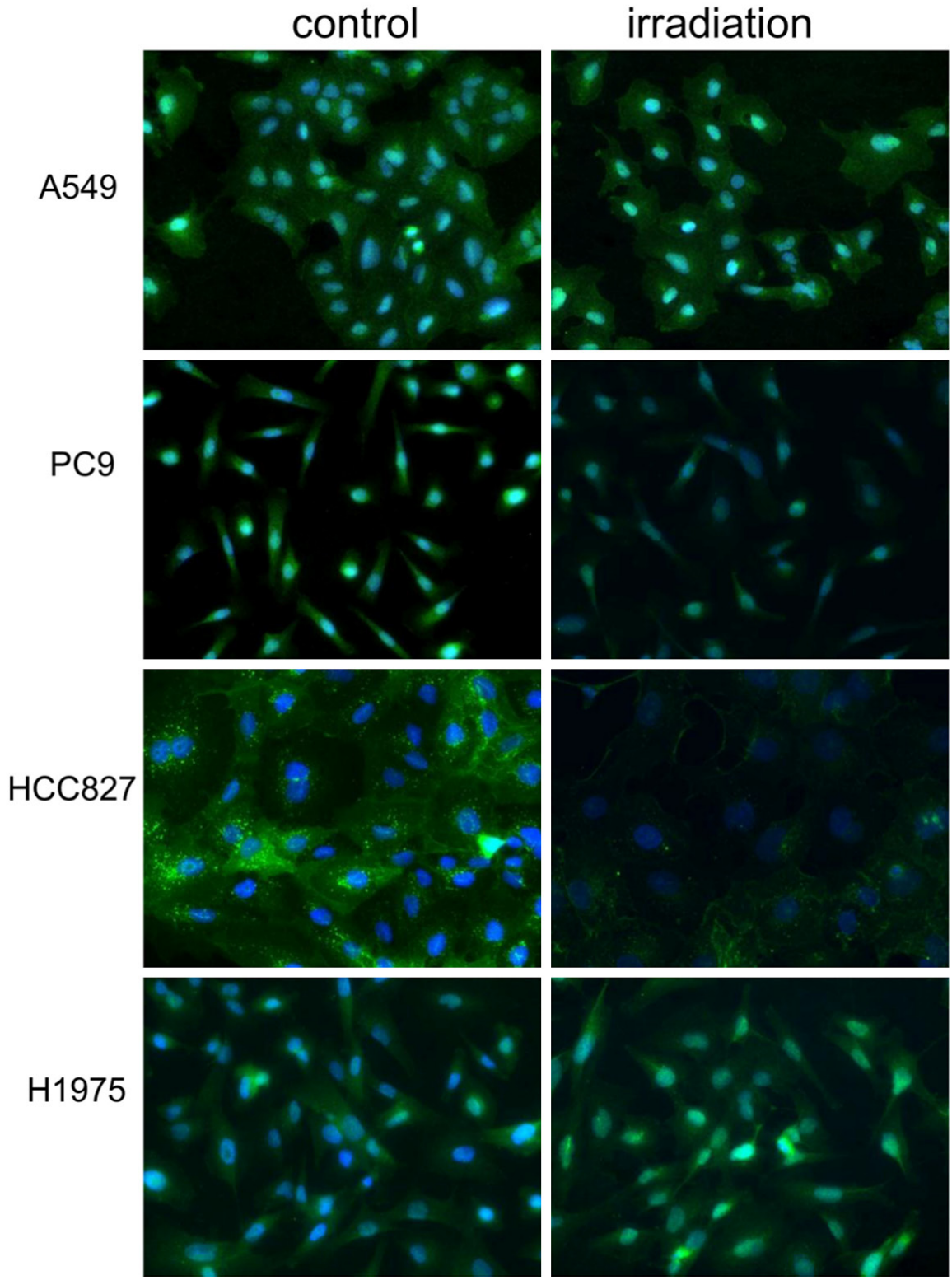

Figure 6. Immunofluorescence staining of EGFR (green) before and after 4 Gy irradiation. Nuclei were stained with DAPI (blue). Irradiation reduced EGFR stain in HCC827 and PC9 cells but did not in A549 and H1975 cells.

observed in all cell lines after the EGF stimulation. Significant levels of phosphorylated EGFR were noted in untreated PC9, HCC827, and H1975 cells. EGF further increased the phos-

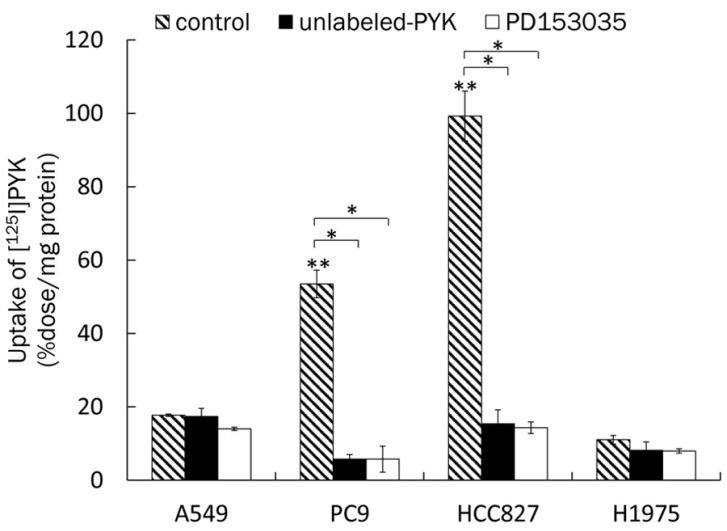

Figure 7. Uptake of [^{125}I]PYK by cultured cells with or without the EGFR-TKI pretreatment. Data represent the mean ± SD, n=4. **P<0.01 were obtained from an ANOVA test by comparing HCC827 and PC9 with A549 and H1975.

phorylation of EGFR in these cell lines and the level of phosphorylation was higher than that in A549 cells with Wt EGFR.

Inhibition of cell proliferation by irradiation and gefitinib

As a result of irradiation, greater decreases in viability were observed in PC9 and HCC827 cells than in A549 and H1975 cells (P<0.01 at 2, 4, 6, 8 Gy for PC9 and P<0.01 at 4, 6, 8 Gy for HCC827 respectively) (**Figure 3**). These results indicated that PC9 and HCC827 cells are more radiosensitive than A549 and H1975 cells. The cell viability curve after irradiation under the effect of gefitinib was shown in **Figure 4**. The decrease of cell viability was more dramatic by the combination of irradiation and gefitinib than irradiation alone in PC9 and HCC827 cells. Gefitinib can increase radiosensitivity in PC9 and HCC827 cells.

Modulation of EGFR expression by irradiation

Expression levels of EGFR mRNA decreased by irradiation, gefitinib treatment and both in PC9 and HCC827 cells (**Figure 5**). Irradiation and/or gefitinib treatment did not reduce EGFR expression significantly in A549 and H1975 cells.

Immunofluorescence analysis also demonstrated that irradiation caused reduction of EGFR stain in PC9 and HCC827 cells (**Figure 6**). In A549 and H1975 cells, however, irradiation did not change EGFR stain significantly.

Uptake of [^{125}I]PYK in cell lines

The uptake of [^{125}I]PYK by PC9 or HCC827 cells was higher than that by A549 or H1975 cells (P<0.01) (**Figure 7**). The pretreatment with non-radioactive PYK or PD153035 inhibited uptake in PC9 and HCC827 cells (P<0.05). Although EGFR was expressed in all 4 cell lines (**Figure 2**), significant binding of [^{125}I]PYK was observed only in PC9 and HCC827 cells. These cells harbored an exon 19 mutation and showed high levels of total and phosphorylated EGFR after EGFR stimulation (**Figure 2**). These results showed that the uptake of [^{125}I]PYK by cells with a special mutation in EGFR was high and specific.

Cell cycle analysis

Cell cycle analysis revealed that the proportion of cells in the G1 phase was significantly higher and the proportion of cells in the S phase was significantly lower in PC9 and HCC827 cells exposed to gefitinib than in untreated cells (P<0.05) (**Figure 8**). Cells in the S phase are resistant to X-ray irradiation. The decrease in the S phase may explain the role of gefitinib as a radiosensitizer in PC9 and HCC827 cells. No significant changes in the proportion of cells in the G1 and S phases were observed for A549 and H1975 cells after the gefitinib treatment.

Discussion

NSCLC cell lines bearing the tyrosine kinase domain mutant of EGFR were shown to be more sensitive to ionizing radiation than wild-type EGFR cell lines [13]. NSCLC cell lines with an EGFR mutation could enhance radiosensitivity by delaying DNA double-strand break (DBS) repair and increasing radiation-induced apoptosis [21]. The findings of our previous study showed that radioiodinated PYK had the potential to evaluate EGFR gene mutations [18, 19]. The present study confirmed that the accumulation of radioiodinated PYK was associated

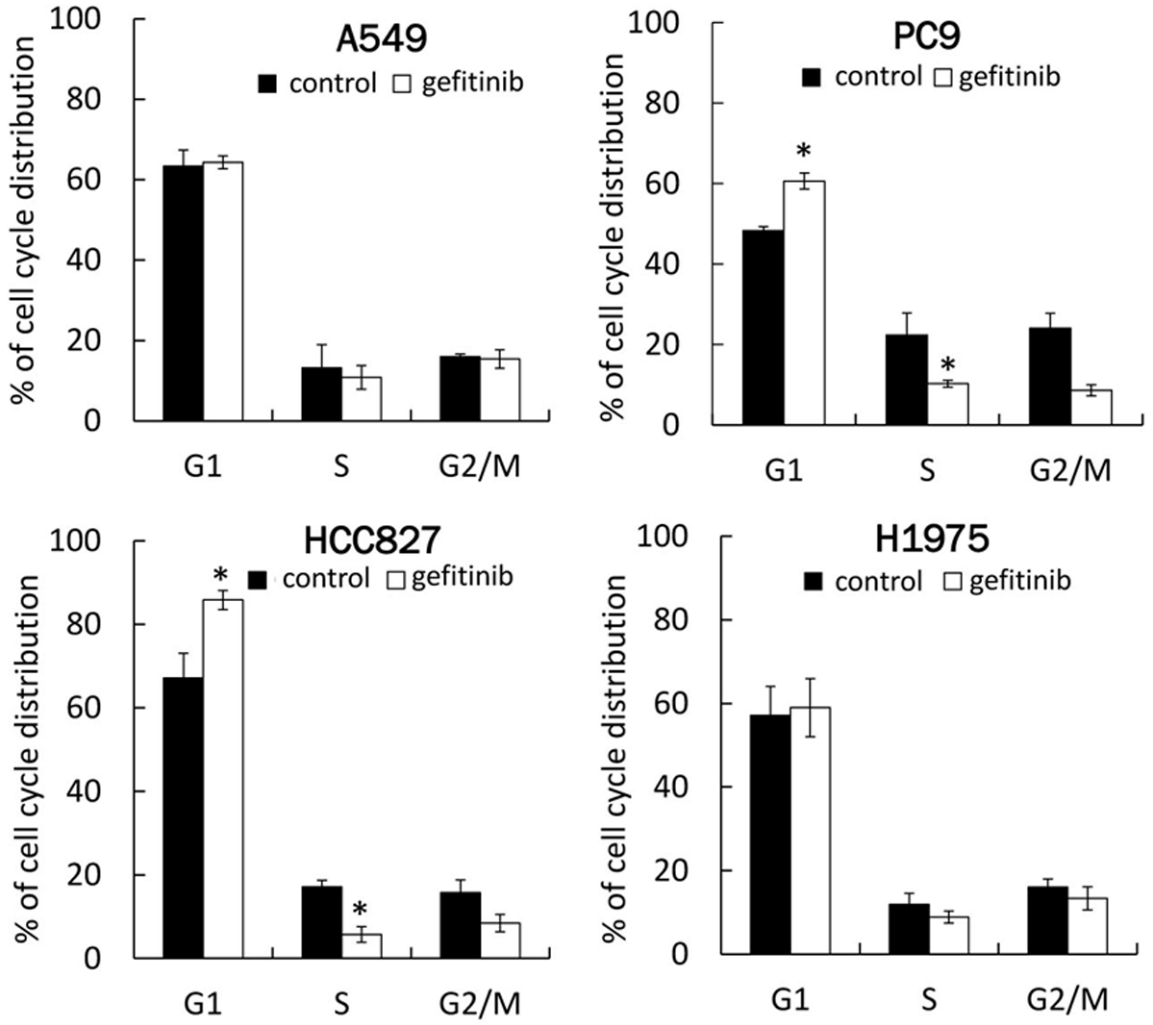

Figure 8. Relative DNA contents in the G1, S, and G2/M phases after the gefitinib treatment in PC9, HCC827, A549, and H1975 cells. Data represent the mean ± SD, n=4. Gefitinib decreased the S phase fraction in PC9 and HCC827 cells, *P<0.05 was obtained from the Student's *t*-test.

with radiosensitivity in NSCLC cell lines. Among the four NSCLC cell lines, PC9 and HCC827 cells that harbored an exon 19 mutation (DelE746A750) were more sensitive to radiation. RT-PCR and immunofluorescence microscopy studies also showed that irradiation caused a more significant effect in PC9 and HCC827 cells than in H1975 and A549 cells.

The uptake of [^{125}I]PYK was higher in PC9 and HCC827 cells than in H1975 and A549 cells (**Figure 7**). Furthermore, the expression and phosphorylation levels of EGFR were high in PC9 and HCC827 cells. The high accumulation of [^{125}I]PYK in PC9 and HCC827 cells may be related to not only the high expression of total and phosphorylated EGFR, but also the exon 19 mutation (DelE746A750) in the EGFR gene. Previous studies demonstrated that an EGFR mutation reduced the affinity of the tyrosine kinase for ATP, while increasing its affinity for EGFR tyrosine kinase inhibitors (EGFR TKIs), such as gefitinib and erlotinib [22]. Since the designed tracer [^{125}I]PYK is a quinazoline derivative, which is similar to gefitinib, the mutation in these cell lines could increase affinity for [^{125}I]PYK. Although H1975 cells had the L858R mutation, it was shown that the second T790M mutation blocked the binding of quinazoline derivatives [22]. This may explain the poorer uptake of [^{125}I]PYK in H1975 cells. The uptake of [^{125}I]PYK in PC9 and HCC827 cells was inhibited by EGFR-TKIs. The specific uptake of [^{125}I]PYK in PC9 and HCC827 cells confirmed that

radioiodinated PYK can be used to detect EGFR mutations.

The present study showed that the number of viable cells after irradiation correlated with the uptake of [^{125}I]PYK. Therefore, radioiodinated PYK may be useful for predicting the radiosensitivity of NSCLC in patients through a nuclear medicine imaging technique. Further studies are needed to determine the feasibility of predicting radiosensitivity with radioiodinated PYK *in vivo*.

Gefitinib enhanced the therapeutic effects of radiation in PC9 and HCC827 cells. The S phase is known to be the most radioresistant phase of the whole cell cycle [23]. DNA-dependent protein kinase (DNA-PK) has been shown to play a key role in DBS repair. The nuclear import and activation of the DNA-PK catalytic subunit (DNA-PKcs) occurs in the S phase [24]. A decrease in the S phase reduces DNA-PKcs, which consequently attenuates DBS repair. G1 cell-cycle arrest could modulate apoptosis and block the cellular repair of radiation-induced damage [25]. A decrease in the S phase fraction and G1 phase arrest by gefitinib may increase radiosensitivity in PC9 cells and HCC827 cells. Because the addition of irradiation to gefitinib was effective in NSCLC cell lines harboring an EGFR mutation, [^{125}I]PYK may also be useful for predicting combination treatments with gefitinib and irradiation.

One of the limitations of our study is the absence of *in vivo* data. Further investigations including *in vivo* studies are in progress. The part of the *ex vivo* biodistribution study using different cell lines was reported previously [19]. We also did not fully investigate the characteristics of these four cell lines. Many factors would involve the radiosensitivity of cells. EGFR mutant status can be one of these factors. We could not disclose stem cell-like characteristics of these cell lines which determine the radiosensitivity.

In conclusion, the uptake of [^{125}I]PYK was closely correlated to the effects of radiation in NSCLC cells. These results suggest that radioiodinated PYK may be useful in predicting the therapeutic effects of radiotherapy in NSCLC patients.

Disclosure of conflict of interest

None to declare.

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