Abstract. The mechanisms underlying lung cancer radioresistance remain to be fully elucidated. The DNA repair pathway is a predominant target of radiotherapy, which is considered to be involved in the acquired radioresistance of cancer cells. The present study aimed to establish a radioresistant cell model using the A549 human lung cancer cell line, and to further investigate the potential mechanisms underlying the radioresistance. The A549R radioresistant lung cancer cell variant was established by exposing the parental A549 cells to repeated γ-ray irradiation at a total dose of 60 Gy. Colony formation assays were then used to determine cell survival following γ-ray exposure. The established radioresistant cells were subsequently treated with or without the NU7026 DNA-PKcs inhibitor. The levels of DNA damage were determined by counting the number of fluorescent γ-H2AX foci in the cells. The cellular capacity for DNA repair was assessed using antibodies for the detection of various DNA repair pathway proteins. The radioresistant sub-clones exhibited significantly decreased survival following NU7026 treatment, compared with the parental cells, as determined by colony formation assays (P<0.05), and this finding was found to be dose-dependent. Treatment with the DNA-dependent protein kinase (DNA-PK) inhibitor significantly reduced γ-H2AX foci formation (P<0.05) following acute radiation exposure in the radioresistant sub-clones, compared with the parental control cells. The decreased levels of γ-H2AX were accompanied by an increase in the percentage of apoptotic cells in the radioresistant cell line following post-radiation treatment with the DNA-PKcs inhibitor. The expression levels of proteins associated with the DNA repair pathway were altered markedly in the cells treated with NU7026. The results of the present study suggested that radioresistance may be associated with enhanced DNA repair following exposure to radiation, resulting in reduced apoptosis. Therefore, the quantity of γ-H2AX determines the radioresistance of cells. The DNA repair pathway is important in mediating radioresistance, and treatment with the DNA-PKcs inhibitor, NU7026 restored the acquired radiation resistance.

Introduction
Radiotherapy is used widely in the treatment of various types of cancer, including non-small cell lung cancer (1). Together with chemotherapy and surgery, radiotherapy has been demonstrated to be extremely efficient. Fractionated radiation is a radiotherapy procedure, which is applied clinically to assist in normal tissue recovery (2). However, the incidence of intrinsic and acquired radioresistance has increased in patients with cancer, resulting in a reduction in treatment success (3). Although efforts have been made to investigate the mechanisms underlying radioresistance, and to identify alternative treatment options, radioresistance remains a significant obstacle in improving lung cancer treatment (4).

The capacity for cells to repair DNA, which is damaged by ionizing radiation is a critical factor in determining cellular radiosensitivity (5). Ionizing radiation induces DNA double strand breaks (DSB), which is considered the most harmful type of DNA damage (6). In mammalian cells, DNA-dependent protein kinase (DNA-PK)-dependent non-homologous end joining (NHEJ) is one of the predominant pathways for repairing DSBs (7). This pathway is initiated by activation of ataxia telangiectasia mutated (ATM) kinase. DNA-PK is a member of the phosphoinositide 3-kinase-like enzyme family, and is a nuclear serine/threonine protein kinase, which is required for repairing DSBs and for D(V)J recombination (8). Cells defend against DNA DSBs via
several repair pathways; among which, the end-joining pathway is the most important (6). There are four proteins, which are involved in this process: DNA-PK, Ku70 and Ku80 (9,10) and XRCC4 (11). ATM is essential for controlling the cellular response to DSB, since it can regulate apoptosis, cell cycle checkpoints and DNA repair signaling processes (12). Following the induction of DSBs, ATM is rapidly activated by intermolecular autophosphorylation (13,14).

The H2AX histone protein is a substrate of ATM kinase, which is required for recruiting DNA repair molecules to sites of damage following exposure to ionizing radiation (15,16). The transformation of histone H2AX at Ser139 to γ-H2AX has previously been recognized as the initial signal step in the response to DNA DSBs. γ-H2AX recruits other proteins to the sites of DSBs and initiates the repair process (17). Suppression of DNA repair may lead to an enhancement of radiosensitivity.

During the DNA repair process, DNA-PK is involved in the NHEJ pathway, and in V(D)J recombination. DNA-PK acts as an enzyme to activate numerous DNA damage-associated proteins and coordinates with ataxia telangiectasia and Rad3 related (ATR) and ATM to phosphorylate proteins associated with DNA damage checkpoints. Previous studies have demonstrated that the inactivation of DNA-PK restores the sensitivity of cells to radiation (18,19). Therefore, an inhibitor targeting DNA-PK has been commercially developed, to provide potential clinical options for the treatment of cancer (20).

The mechanisms by which irradiated cells sense DNA damage remain to be elucidated, however, it is likely that DNA-PK and ATM are major signal transducers. Since the crucial DNA repair enzymes are pivotal in cancer research, the discovery of their inhibitors is of significant interest. The present study aimed to investigate the effects of the novel specific DNA-PKcs inhibitor, NU7026, which has been reported to act as a radiosensitizer in vitro (21,22), combined with ionizing radiation, on the activation of several components of the DNA damage and repair response signaling pathway. In addition, the present study also aimed to determine its effect on the induction of apoptosis in acquired radioresistant cells.

Materials and methods

Cells and cell culture. The A549 human lung cancer cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in 25 ml RPMI 1640 with modified Dulbecco's modified Eagle's medium, supplemented with 20% fetal calf serum, 0.05% L-glutamine, 150 IU/ml penicillin and 50 µg/ml streptomycin (all from Sigma-Aldrich, St. Louis, MO, USA), at 37°C in a humidified atmosphere containing 5% CO2. The cell culture medium was regularly replaced, and the cells were separated once they had reached 80% confluence. A maximum of 20 passages were used. To avoid mycoplasma contamination, the cell line was assessed monthly using polymerase chain reaction (PCR) (23). For PCR, the ATCC Universal Mycoplasma Detection Kit (ATCC) was used. In brief, 10 µl reaction mixture with 10 ng sample was analyzed, using the following primer sequences: Forward 5'-GGGAGCAAAACAGGATTAG ATACCCT-3' and reverse 5'-TGCAACCATCTGTCACTCT GTTAAACCTC-3'. The PCR cycling conditions were as follows: 32 cycles of 4 sec at 95°C, 8 sec at 65°C and 16 sec at 72°C with the Applied Biosystems GeneAmp 9600 thermal cycler (Applied Biosystems Life Technologies, Foster City, CA, USA). The cell lines were authenticated using Short Tandem Repeats (STR) analysis to avoid errors in identification, which used the GenePrint 10 system (Promega Corporation, Madison, WI, USA) to detect TH01, TPOX, VWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317, D5S818 and D21S11 comparing with the Applied Biosystems GeneAmp 9600 thermal cycler.

Development of acquired radioresistant cells. A step-wise method (24) was used to produce the radioresistant cell line. Parental A549 cells (1x106) were plated in a 75 cm2 culture flask with 25 ml RPMI 1640. Once the cells had reached 70% confluence, the flask was placed in a Theratron Cobalt-60 unit (Best Theratronics, Ontario, Canada) and irradiated with 4 Gy γ-rays, at a dose rate of 1 Gy per minute. The culture medium was replaced immediately following irradiation, and regular medium replacement and separation of the cells was performed when the cells had reached 90% confluence. To separate the cells, they were washed with ice-cold phosphate-buffered saline (PBS; Sigma-Aldrich), then 0.5 ml trypsin (Sigma-Aldrich) was added into the culture flask, which was incubated at 37°C in a humidified atmosphere with 5% CO2 for 5 min. The adherent cells were removed from the dish with a cold plastic cell scraper and 5 ml RPMI 1640 was added. A total of 1 ml cell suspension was carefully added into a fresh flask with 24 ml RPMI 1640, which was then incubated at 37°C in a humidified atmosphere containing 5% CO2. Irradiation was performed a total of 13 times (increasing doses starting at 4 Gy, with a total exposure of 60 Gy) within 6 months. The parental cells were cultured in the same manner without irradiation.

In vitro γ-ray irradiation. The cells were counted using a BC TC20 Automatic Cell Counter (BD Biosciences, San Jose, CA, USA) and adjusted to a density of 1x106 cells/ml. The cells were then placed in a 5% CO2 incubator at 37°C, and γ-ray irradiation was performed using a Theratron Cobalt-60 treatment unit at a dose rate of 1 Gy/min.

Colony formation assay. The cultured A549 parental and A549R radioresistant cells (1x106) were irradiated with a single dose of 0, 4, 8 and 12 Gy. Following irradiation, the cells were plated into 6 cm dishes and cultured with 25 ml RPMI 1640 in an incubator at 37°C with a humidified atmosphere containing 5% CO2 for 2 weeks with no additional treatment. Colony formation was visualized by staining with 0.02% crystal violet solution (w/v) in 75% ethanol; Sigma-Aldrich), and the colonies were counted. The surviving fraction was then calculated in the two groups, by first calculating the plating efficiency [PE; (number of colonies counted/number of cells plated)x100%] for the treatment and control groups. The surviving fraction (SF) could then be calculated; SF = (PE of treated group/PE of control group) x100%.

Apoptosis assay. Subsequent to trypsinization and incubation at 37°C for 24 h, the A549 and A549R cells (1x106) were seeded in 10 mm dishes and incubated overnight at 37°C.
Following irradiation with 4, 8 and 12 Gy, the cells were treated with the DNA-PK catalytic subunit (cs) inhibitor NU7026 (Sigma-Aldrich), at a concentration of 1 µM for 24 h. Following 24 h incubation, apoptotic analyses were performed using a flow cytometer (BD FACSCalibur; BD Biosciences) with propidium iodide (PI) and annexin V staining (Invitrogen Life Technologies, Carlsbad, CA, USA), as described previously (19). In brief, cells were washed in cold PBS then were treated with annexin-binding buffer. Suspended cells were then fixed in 70% cold ethanol and treated with 10 g/l RNase, then 5 µl Alexa Fluor 488 annexin V and 1 µl PI (100 µg/ml) were added per 100 µl cell suspension. Following incubation for 15 min at room temperature, 400 µl annexin-binding buffer was added with gentle mixing. Cells with early apoptotic signals (stained with annexin V) and cells with late apoptotic signals (stained with PI) were quantified and analyzed, and each assay was performed in triplicate.

The distributions of apoptotic cells were analyzed using ModFit LT™ software, version 4.0 (Verity Software House, Topsham, ME, USA).

γ-H2AX foci formation. The cells were trypsinized and resuspended following fixation with 2% paraformaldehyde (Sigma-Aldrich) for 15 min, and were subsequently permeabilized with 0.5% Triton X-100 (Sigma-Aldrich). The cells were then incubated with a mouse monoclonal primary antibody specific for Ser 139 phosphorylation of H2AX (ab11174; Abcam, Cambridge, MA, USA; 1:400) at 4°C overnight, followed by incubation with the polyclonal goat anti-mouse Alexa Fluor 488-labeled secondary antibody (A-10667; Life Technologies, Grand Island, NY, USA; 1:1,000) for 2 h at room temperature. The cells were then incubated with primary antibodies at 4°C overnight. Following washing with buffer solution, the membranes were incubated with secondary antibody overnight. The protein bands were visualized using Kodak film (Eastman Kodak, Rochester, NY, USA) in a dark room.

Western blotting. All procedures were conducted according to the manufacturer's instructions (Invitrogen Life Technologies). Equal quantities of proteins (50 µg) were obtained from the two cell groups using 0.5 ml ice-cold lysis buffer (Invitrogen Life Technologies), maintaining constant agitation for 30 min at 4°C, and centrifuging in a microcentrifuge at 4°C for 20 min at 14,000 x g. The protein samples were separated by SDS-PAGE (10% gel for the upper chamber and 5% for the lower chamber; Invitrogen Life Technologies), alongside molecular weight markers, and were then electrotransferred onto nitrocellulose membranes (Life Technologies). The membranes were blocked for 1 hour at room temperature, or overnight at 4°C using 5% blocking solution (1X Tris-buffered saline with Tween 20 and nonfat dried milk; Invitrogen Life Technologies). The membranes were then incubated with primary antibodies at 4°C overnight. Following washing with buffer solution, the membranes were incubated with secondary antibody overnight. The protein bands were visualized using Kodak film (Eastman Kodak, Rochester, NY, USA) in a dark room.

Statistical analysis. Data are expressed as the mean ± standard error of the mean, and all experiments were performed in triplicate. SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used to perform all statistical analyses. Student's t-test and one-way analysis of variance were used to analyze the differences between the groups. P<0.05 was considered to indicate a statistically significant difference.
**Results**

**Identification of established radioresistant A549 cells.** To generate radioresistant cells, the parental A549 cell line was irradiated for 5 months using a step-wise method, with an accumulated dose of 60 Gy. The A549R acquired resistance cell line was then maintained in culture medium for 10 passages without radiation. No significant changes in cell morphology were observed in the A549R cells following irradiation, compared with the parent cells (Fig. 1A), under microscopy.
The colony formation assay revealed that the A549R cells exhibited higher rates of cell survival rate, compared with the parental cells following irradiation (Fig. 1B).

**DNA-PKcs inhibitor treatment induces apoptosis and reduces survival of radioresistant cells following irradiation.** The radiosensitivities of the A549R cells with or without NU7026 were compared using a colony formation assay (Fig. 2A). The survival fraction was decreased in the A549R cells treated with NU7026 following radiation therapy at different doses, indicating that the radioresistance of the A549R cells was reversed following treatment with the DNA-PKcs inhibitor. As shown in Fig. 2B, the percentage of radiation-induced apoptotic cells was markedly increased in the NU7026-treated cells, indicating the potential stimulatory effects of NU7026 on apoptosis when used in conjunction with irradiation.

**DNA damage signal is decreased following treatment with a DNA-PKcs inhibitor.** The effect of the NU7026 DNA-PKcs inhibitor were evaluated on the cells with acquired radioresistance treated with different doses of irradiation (0-12 Gy) within 24 h. The level of DNA damage induced by irradiation was determined by counting the number of histone γ-H2AX foci under fluorescence microscopy. A total of 500 nuclei were counted in each group (Fig. 3A). The formation of γ-H2AX was significantly downregulated following treatment with NU7026 and 8 Gy or 12 Gy irradiation (Fig. 3B). These results suggested that treatment with NU7026 suppressed the radiation-induced DNA repair signals in the A549R cells following high dose irradiation.

**Discussion**

The results of the present study provided evidence for the radiosensitizing role of NU7026 in lung cancer cells exhibiting acquired radioresistance. As expected, treatment with the DNA-PKcs inhibitor directly attenuated the ability of the cells to repair DNA, by inactivating radiation-induced NHEJ pathway proteins, and further increased the levels of radiation-induced apoptosis. This inhibitory effect was
associated with initially decreased levels of the γ-H2AX DNA repair factor and impaired the recruitment of other repair proteins to the DNA DSB site. This resulted in the restoration of radiosensitivity in the resistant cells treated with irradiation, and a reduction in cell survival. These results suggested the potential therapeutic application of NU7026 in the treatment of radioresistant cancer cells.

Theoretically, the ability of a cell to repair radiation-induced DNA damage determines the radiosensitivity of the cell (25). The more proteins, which are associated with DNA repair, and accumulate at the break site, the less radiation-induced damage that occurs (11). Therefore, due to its ability to recruit other cell repair proteins, γ-H2AX formation is regularly used to assess the level of cellular DNA damage (26). γ-H2AX can be detected by either flow cytometry or immunofluorescence, which quantify the percentage of γ-H2AX formation. Increased levels of γ-H2AX lead to enhanced DNA repair ability (27). The present study demonstrated that treatment with NU7026 had an effect on the levels of radiation-induced γ-H2AX formation. Decreased levels of γ-H2AX resulted in a reduction in DSB signals, which led to increased cell apoptosis and decreased cell viability following irradiation.

DNA-PKcs is a key component of the NHEJ pathway, and is important in DNA DSB repair, genomic integrity and maintenance of telomere stability (28,29). DNA-PKcs is upregulated in various types of cancer (30,31). It has also been reported that increased expression of DNA-PKcs and kinase activity are closely associated with radioresistance or chemoresistance (32). Inhibitors of DNA-PKcs, including NU7441, have been developed to enhance local tumor control (33). The present study demonstrated that adjuvant treatment with NU7026 overcame radioresistance in lung cancer cells, by reducing the γ-H2AX signal. The NU7026-mediated radiosensitization in lung cancer cells was predominantly associated with delayed radiation-induced DSB repair. A previous study reported that a DNA-PKcs inhibitor was able to enhance the glioma cell apoptosis percentage with irradiation treatment (8).

The present study demonstrated markedly altered expression levels of DNA repair pathway proteins in the NU7026-treated cells following irradiation. As a key factor in DNA repair, the phosphorylation of DNA-PKcs at Thr2609 inhibited the recruitment of repair molecules localized in the DNA DSB region, attenuating the cell repair defense system. In addition, two key subunits of DNA-PKcs, Ku70 and Ku80, exhibited reduced protein expression levels in cells treated with NU7026, in addition to the inhibitory effect of NU7026 on the DNA-PKcs. These results suggested that the effect of NU7026 on DNA-PKcs functions through suppression of its inactivation (34).

In conclusion, treatment with a DNA-PKcs inhibitor successfully restored radiosensitivity in lung cancer cells with acquired radioresistance. According to the results of the present study, the possible underlying mechanism may have been the inactivation of NHEJ components by the DNA-PKcs inhibitor, which may have subsequently impaired DNA repair ability, enhanced apoptosis and decreased cell viability. These findings approved the step-wise method as a useful technique to generate a resistant cell model, and revealed the potential application of a DNA-PKcs inhibitor on acquired radioresistance in lung cancer.

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References


