

# PTEN/pAkt/p53 signaling pathway correlates with the radioresponse of non-small cell lung cancer

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**Abstract.** The sensitivity or resistance of cancer cells and normal tissues to ionizing radiation plays an important role in the clinical setting of lung cancer treatment. However, to date the exact molecular mechanisms of intrinsic radiosensitivity have not been well explained. In this study, we compared the radiosensitivity or radioresistance in two non-small cell lung cancers (NSCLCs), H460 and A549, and investigated the signaling pathways that confer radioresistance. H460 cells showed a significant G<sub>2</sub>/M arrest after 12 h of irradiation (5 Gy), reaching 60% of G<sub>2</sub>/M phase arrest. A549 cells also showed a significant G<sub>2</sub>/M arrest after 12 h of exposure; however, this arrest completely disappeared after 24 h of exposure. A549 has higher methylated CpG sites in PTEN, which is correlated with tumor radioresistance in some cancer cells, than H460 cells, and the average of the extent of the methylation was ~4.3 times higher in A549 cells than in H460 cells. As a result, PTEN expression was lower in A549 than in H460. Conducting Western blot analysis, we found that PTEN acted as a negative regulator for pAkt, and the pAkt acted as a negative regulator for p53 expression. According to the above results, we concluded that the radiosensitivity shown in H460 cells may be due to the higher expression of PTEN through p53 signaling pathway.

## Introduction

Of the numerous public health problems, lung cancer is one of the leading causes of death throughout the world (1-3). Patients with lung cancer have a <15% rate of survival over 5 years with current therapeutic modalities; this statistic has changed only minimally in the last 25 years, underscoring the need for new therapeutic strategies. Thus, understanding the molecular mechanisms involved in the pathogenesis of lung cancer may

provide greater opportunities to improve therapeutic methods for this fatal disease (4,5).

For almost 100 years, radiotherapy with ionizing radiation, either alone or in combination with chemotherapy, has been widely used, but radioresistance of some human tumors to ionizing radiation and injury to normal tissues are the primary disadvantages that have an impact on the curative effect of clinical radiotherapy (6). The sensitivity or resistance of cancer cells and normal tissues to ionizing radiation plays an important role in the clinical setting. However, to date the exact molecular mechanisms of intrinsic radiosensitivity have not been clarified. Numerous oncogenes and anti-oncogenes (tumor-suppressor genes) seem to be responsible for the intrinsic radiosensitivity of a tumor and their cross-talk plays an important role (6). Oncogenes, such as *ras*, *myc*, *raf*, *cox-2*, *PTEN*, *PI3K/Akt*, and mutated *p53*, are correlated with tumor radioresistance in many cells, whereas the most important tumor-suppressor gene, wild-type *p53*, is correlated with tumor radiosensitivity (7-27).

Although chemotherapy is often used to treat patients with non-small cell lung cancer (NSCLC), it offers only a small improvement and cell lines derived from these tumors exhibit an intrinsic resistance to both chemotherapy and radiotherapy *in vitro* compared with other types of cancer cells (28). In addition, great differences in response exist between NSCLCs with the same histology (29). Therefore, it is necessary to determine appropriate strategies to improve treatment efficacy, especially with respect to a curative approach in radiotherapy (30,31). In this study, we compared the radiosensitivity or radioresistance in two NSCLCs, H460 and A549, which have a functional *p53*, and investigated the signaling pathways that confer radioresistance.

## Materials and methods

**Cell culture and irradiation.** All NSCLC cell lines used in the study were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Waltham, MA, USA) and penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 95% air/5% CO<sub>2</sub>. Cells were inoculated at a density of 1x10<sup>5</sup> cells in a T-25 flask and incubated for 1 day, followed by irradiation with a dose of 10 Gy using a <sup>60</sup>Co γ-ray at a dose rate of 0.2 Gy/min.

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**cDNA synthesis and PCR amplification.** Total RNA was isolated from H460 and A549 using the Qiagen RNA extraction kit (Qiagen, Valencia, CA, USA). To generate first-strand cDNA from the total RNA (1  $\mu$ g) using oligo dT, a cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada) was used. Resultant cDNAs served as templates for PCR amplification with forward and reverse primers as follows: PTEN-F, 5'-ACGGGAAGACAAGTTCATGTAC; PTEN-R, 5'-ACAGTAGAGGAGCCGTCAAA;  $\beta$ -actin-F, 5'-ATGTGCAAGGCCCGCTTCG;  $\beta$ -actin-R, 5'-TTAATGTCACGCACGATTCC. For amplifying GC-rich PTEN template high, we added a specific buffer, i-GC capture solution (Intron Biotechnology, Korea), to the PCR mixture. The PCR conditions for PTEN were: denaturing at 95°C for 30 sec, followed by 35 cycles at 95°C for 30 sec, at 58°C for 30 sec, at 72°C for 1 min, and a final extension at 72°C for 5 min. The amplified PCR products were analyzed by agarose gel (1%) electrophoresis, and photographed under UV light.

**Western blot analysis.** Anti-p53 and anti-PTEN antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Akt, phospho-Akt (pAkt), and  $\beta$ -actin were purchased from Cell Signaling Technology (Beverly, MA, USA). All reagents used in this study were reagent grade or better. Protein concentration was determined using a Lowry kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were separated on 12% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia, Piscataway, NJ, USA). The blots were blocked for 2 h at room temperature with blocking buffer [10% nonfat milk in PBS buffer containing 0.1% Tween-20 (TBS)]. The membrane was incubated at room temperature for 1 h with specific antibodies. After washing with TBS, the membrane was incubated with a horseradish peroxidase-labeled secondary antibody and visualized using the Westzol enhanced chemiluminescence detection kit (Intron Biotechnology, Gyungki-do, Korea).

**Flow cytometric detection.** Irradiated and non-irradiated cells were collected, washed with PBS, and fixed with 70% ethanol at 4°C for 2 h in the dark. Fixed cells were washed with PBS and stained with propidium iodide (50  $\mu$ g/ml). The DNA content was measured with a FACScan (EPICS XL, Beckman Coulter Counter, Fullerton, CA, USA). A minimum of 10,000 cells was counted for each sample. The percentage of cells in each cell phase was determined by Phoenix Multi-cycler Software (Phoenix Flow System, San Diego, CA, USA).

**Sodium bisulfite modification.** Bisulfite-modified genomic DNA (gDNA) was prepared using the EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. The bisulfite reaction was carried out on 400 ng gDNA, the reaction volume was adjusted to 20  $\mu$ l with sterile water, and 130  $\mu$ l CT conversion reagent was added. The sample tubes were placed in a thermal cycler (MJ Research, Waltham, MA, USA) and the following steps were performed: 10 min at 98°C and 2 h 30 min at 64°C, and then stored at 4°C. The resultant DNA was purified using reagent contained in EZ DNA Methylation-Gold kit (Zymo Research). The converted samples were added to a Zymo-Spin

IC™ column containing 600  $\mu$ l of the M-Binding buffer and mixed by inverting the column several times. The column was centrifuged at full speed for 30 sec and the flow-through discarded. The column was washed by adding 200  $\mu$ l M-Wash buffer and centrifuged at full speed and then 200  $\mu$ l M-desulphonation buffer was added to the column and incubated at room temperature (20-30°C) for 15-20 min. After incubation, the column was centrifuged at full speed for 30 sec. The column was washed by adding 200  $\mu$ l M-Wash buffer and centrifuged at full speed (this step was repeated once more). The converted gDNA was eluted by adding 20  $\mu$ l M-Elution buffer into the column. DNA samples were finally stored at -20°C until further use.

**Pyrosequencing analysis.** PCR reaction was carried out in a volume of 50  $\mu$ l with  $\leq$ 20 ng converted gDNA, 5  $\mu$ l 10X Taq buffer, 5 U Hot/Start Taq polymerase (Enzynomics, Daejeon, Korea), 4  $\mu$ l of each 2.5 mM dNTP mixture, 2  $\mu$ l of 10 pmol/ $\mu$ l Primer-S, and 2  $\mu$ l of 10 pmol/ $\mu$ l biotinylated-Primer-As. The amplification was carried out according to the general guidelines suggested by pyrosequencing: denaturing at 95°C for 15 min, followed by 45 cycles at 95°C for 40 sec, at 55°C for 40 sec, at 72°C for 40 sec, and a final extension at 72°C for 10 min. PCR (5  $\mu$ l) was confirmed by electrophoresis in a 3% agarose gel and visualized by ethidium bromide staining. A ssDNA template was prepared from 20-25  $\mu$ l biotinylated PCR product using streptavidin Sepharose® HP beads (Amersham Biosciences, Uppsala, Sweden) following the PSQ 96 sample preparation guide using multichannel pipettes. Then 15 pmol of the respective sequencing primer set was added for analysis. Sequencing was performed on a PyroMark ID system with the Pyro Gold reagents kit (Biotage, Charlottesville, VA, USA) according to the manufacturer's instructions without further optimization. The methylation percentage was calculated by the average of the degree of methylation at 5 or 6 CpG sites formulated in pyrosequencing.

**Microscopy and analysis of cell survival fraction.** To compare the differences of morphology between H460 and A549, cells were visualized by light microscopy (Leica Microsystems, Westchester, IL, USA). Images were captured with a Cannon digital camera system (model: Power Shot S45).

## Results

Comparison of G<sub>2</sub>/M arrest and cytotoxicity in H460 and A549. We first compared the change of cell cycle in two NSCLCs, H460 and A549, after exposing cells to  $\gamma$ -radiation. Results of typical experiments are shown in Fig. 1A and B. H460 cells showed a significant G<sub>2</sub>/M arrest after 12 h of irradiation, reaching up to 60% of G<sub>2</sub>/M phase arrest. A549 cells also showed a significant G<sub>2</sub>/M arrest after 12 h of exposure; however, this disappeared after 24 h of exposure. Moreover, irradiated H460 cells showed a significant cytotoxicity, unlike A549 cells that showed great tolerance to radiation at 10 Gy (Fig. 2A). This lower cell survival in H460 cells may come from cell detachment, because numerous floating cells (indicated by arrows) in the region of supernatant appeared in the irradiated H460 cells (Fig. 2B). In contrast, no floating cells were seen in the irradiated A549 cells.

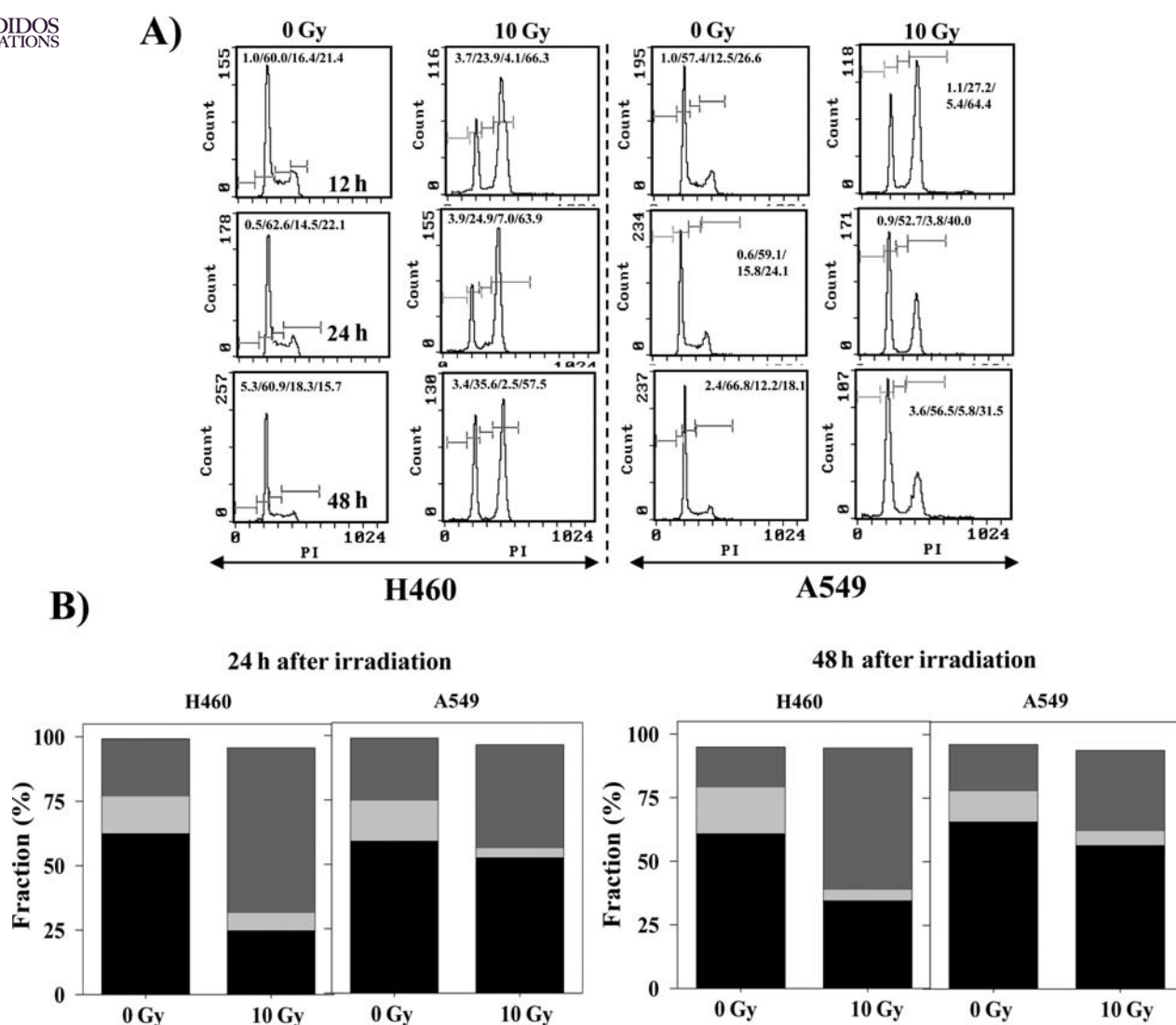


Figure 1. Induction of G<sub>2</sub>/M arrest in H460 by irradiation. (A) FAScan analysis in H460 and A549. (B) Comparison of each cell cycle fraction in H460 and A549. Dark gray, G<sub>2</sub>/M phase; bright gray, S phase; black, G<sub>1</sub> phase.

*Analysis of methylation in the region of CpG islands in H460 and A549.* As previously noted, PTEN is correlated with tumor radioresistance in certain cancer cells. Thus, we investigated whether PTEN participates in the signaling pathway for radioresistance in the two NSCLCs. We used the bisulfite pyrosequencing method for methylation analyses of the *PTEN* gene. Each primer was designed using PSQ assay design program (Biotage), and the sequences of primers are listed in Fig. 3A. We selected five CpG sites (positions 1-5) as shown in Fig. 3A, and bisulfite-modified gDNA was prepared using EZ DNA Methylation-Gold kit (Zymo Research) as noted in Materials and methods, in which the Y sequence (depicted as boxes) means the methylated site in H460 and A549 through positions 1-5. After conducting PCR amplification, the methylation percentage was calculated by averaging the degree of methylation at 5 CpG sites formulated in pyrosequencing (Fig. 3B). As shown in Fig. 3C, the higher methylated CpG sites in all the positions tested were detected in A549 cells rather than in H460 cells, and the average of the extent of the methylation was ~4.3 times higher in A549 cells than in H460 cells. We also confirmed the expression level of PTEN in

H460 and A549 cells by RT-PCR analysis. As shown in Fig. 3D, PTEN was highly expressed in H460, but not in A549 cells.

*Determination of the signaling pathways involved in radio-response.* A signaling pathway for PTEN through p53 has been established. We investigated whether the pathway would be associated with the differences in radioresistance in both NSCLCs. As expected, PTEN acts as a negative regulator for pAkt, and the pAkt acts as a negative regulator for p53 expression (Fig. 4). We concluded that the radiosensitivity shown in H460 cells may be due to the PTEN expression through p53 signaling pathway.

## Discussion

The combination of modalities for cancer treatment offers improvements in the survival of patients compared with individual therapeutic approaches (32). These therapeutic benefits have been achieved with combinations of chemotherapy and radiotherapy in a variety of cancers (32). The

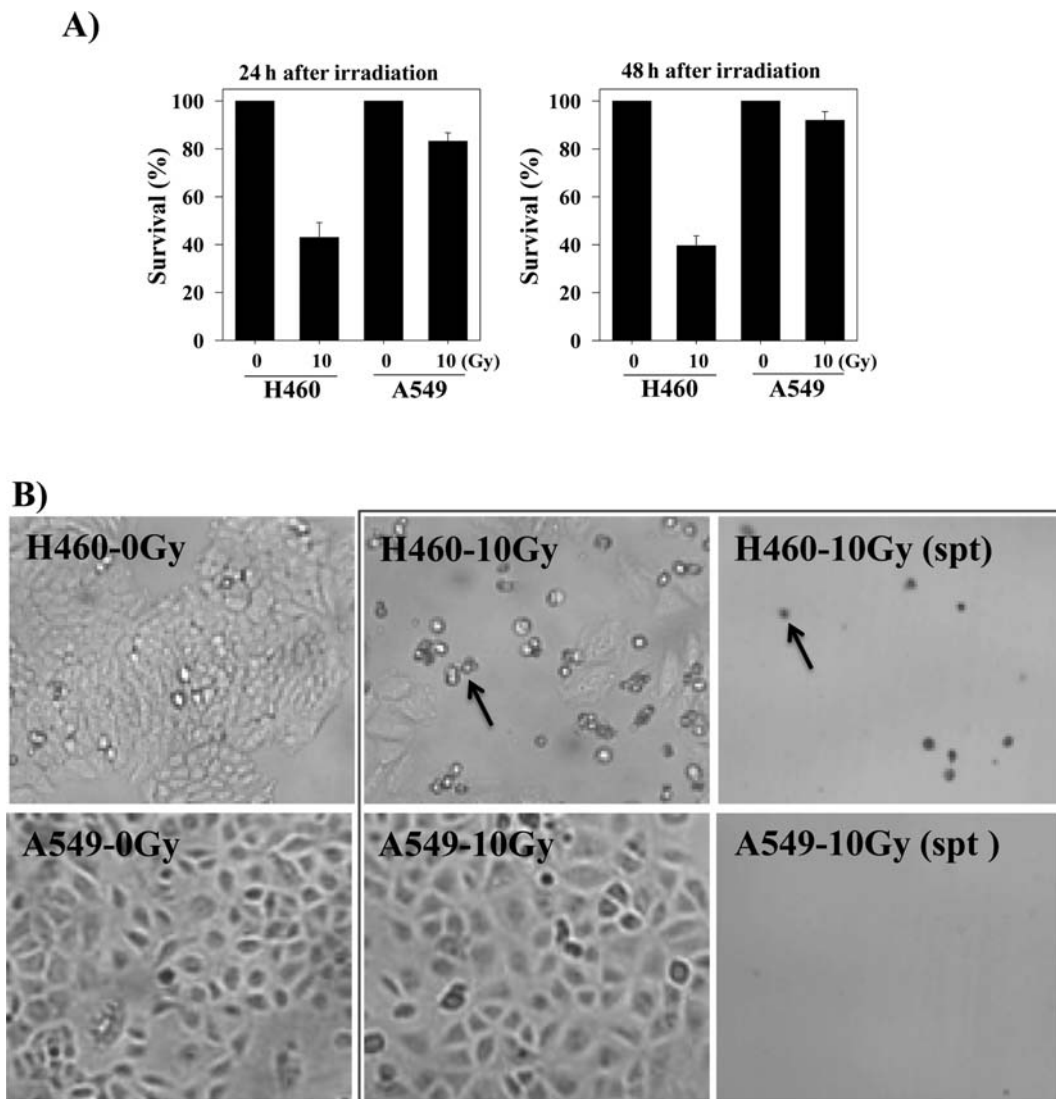


Figure 2. Comparison of cell cytotoxicity. (A) Comparison of surviving fractions after irradiation in H460 and A549 cells. (B) Microscopic views after irradiation. Cells after irradiation were harvested and resuspended in PBS. Floating cells from both total cell culture and the supernatant (spt) were photographed and are indicated by arrows.

cytotoxicity of most chemotherapeutic agents as well as that of radiation is highly dependent on the phase of the cell cycle; for example, the ability of anti-microtubule agents to block cell cycle progression in the G<sub>2</sub>/M phase is the biologic basis for combination of these agents with radiation (32,33). The G<sub>2</sub>/M phase is the one most sensitive to ionizing radiation; thus, many research efforts have focused on the combination of radiotherapy methods that arrest cells in the G<sub>2</sub>/M phase by using specific chemicals and then irradiating the cells to increase the radiosensitivity potential (11-13,34,35). However, although differences in radioresponse between various small cell lung cancers (SCLCs) and NSCLCs are known (29), little has been determined between different NSCLCs.

One factor known to increase tumor cell resistance to radiation, the importance of PI3K/AKT targeting for overcoming resistance of tumors to radiotherapy, has been tested *in vitro* and *in vivo* and the results indicate this cascade is a promising target (24-26). The activation of Akt plays a critical

role in fundamental cellular functions such as cell proliferation and survival by phosphorylation of a variety of substrates (36-38). Constitutively active Akt results in augmented resistance against apoptotic cellular insults, such as growth factor deprivation, UV irradiation, or loss of matrix attachment (39). Akt activation is found in many types of human tumors including breast cancer, lung cancer, melanoma, and leukemia (40,41).

Human p53 is a 393-amino acid nuclear protein that acts biochemically as a transcription factor and biologically as a tumor suppressor (42). As a key regulator of cell growth and cell death, p53 is activated by many environmental stimuli, including DNA-damaging agents. Activated p53 acts as a guardian of the genome by inducing growth arrest to allow cells to repair the damage or apoptosis if the damage is too severe and irreparable (43,44). Thus, it is no surprise that p53 is frequently inactivated via multiple mechanisms during human carcinogenesis. The most common mechanism is the point mutation at the *p53* gene, which occurs in 50% of human

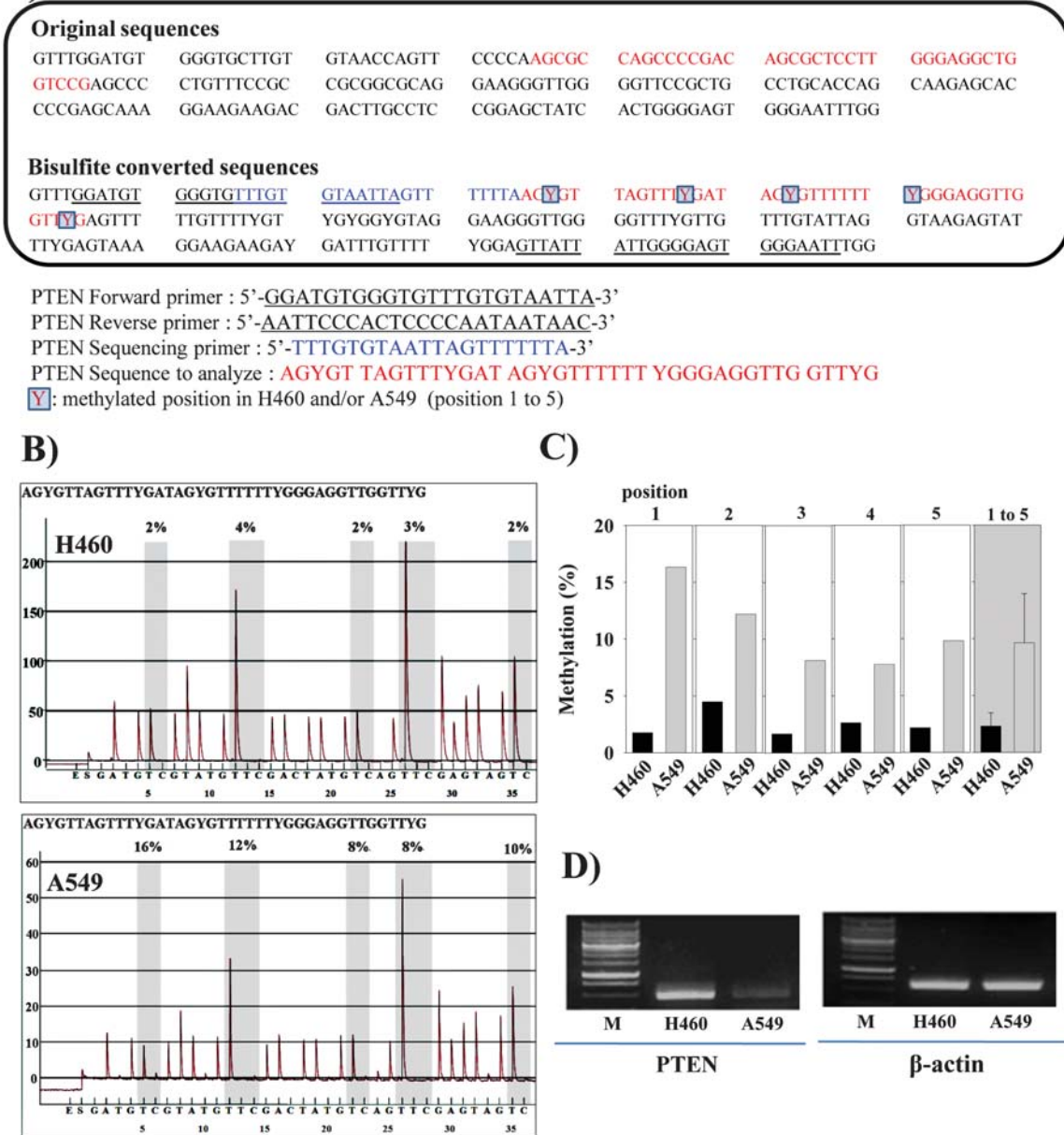


Figure 3. Pyrosequencing of 5 CpG islands on PTEN. (A) Original sequence of PTEN and bisulfite-converted sequences. Each Y shown in red sequences indicates the methylated position in both cell lines. (B) Diagram of pyrosequencing. Each colored box indicates the position of the five Ys shown in panel A. (C) Comparison of fractions in the methylated CpG positions. The last bars indicate the means of all the methylated positions, 1-6. (D) RT-PCR analysis of PTEN genes in H460 and A549 cells.

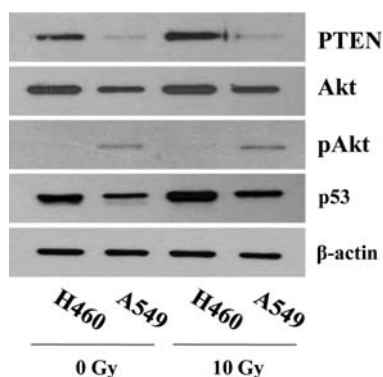


Figure 4. Western blot analysis. Cells were grown for 48 h after irradiation and harvested.

cancers (45). Moreover, in certain cancer cells that bear wild-type p53, p53 is often inactivated by a variety of mechanisms. One common mechanism is Mdm2-mediated p53 binding, ubiquitination, and degradation (46). As noted above, p53 status and cancer radiosensitivity have been widely studied. Loss of p53 function, as in p53 mutation, increases resistance to radiation in many human cancers (7-13,34,47).

In this study, we found that the A549 cells were more resistant compared to H460 cells. We also found that p53 expression was increased by radiation, meaning that cells encountered severe stress due to irradiation. However, approaches to endure the stress were somewhat different in the two NSCLCs. The extent of p53 induction was lower in A549 than in H460 and this difference in p53 induction by irradiation must be the determinant for the radiosensitivity in

both lung cancer cell types. The decreased expression level of p53 in A549 compared to in H460 must come from the higher induction of pAkt in A549 than in H460 according to the Western blot experiments. Pyrosequencing has been widely used for determining the content of the methylated CpG island in many genes with a high GC rate. Thus, we compared the extent of methylation between two PTEN genes, known to be negative regulators of pAkt, existing in two cancer cell types. As expected, all the selected 5 CpG positions in PTEN were more methylated in A549, and we concluded that the transcriptional expression of the PTEN gene in A549 was lower than in H460. In conclusion, the radioresistance that has been shown in A549, results from the highly methylated PTEN.

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